RENAL FUNCTION AND DISEASE IN ZEBRAFISH

DISSERTATION

ZUR

ERLANGUNG DER NATURWISSENSCHAFTLICHEN DOKTORWÜRDE

(Dr. sc. nat.)

VORGELEGT DER

MATHEMATISCH-NATURWISSENSCHAFTLICHEN FAKULTÄT

DER

UNIVERSITÄT ZÜRICH

VON

YUYA SUGANO

AUS

JAPAN

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ZÜRICH, 2015

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I PREFACE

"*Life is like a box of chocolate.*

- you never know what you're gonna get"

- Forrest Gump

II SUMMARY

The kidney maintains homeostasis of our internal environment. In this thesis, the zebrafish was exploited as a model system to study human renal function and disease. The zebrafish is a useful vertebrate model for human kidney disease with an array of powerful genetic tools. The zebrafish pronephros is a simple and easily accessible system, yet with a high degree of conservation with the mammalian metanephric nephrons. Research presented here consists mainly of two parts; the glomerulus and the distal tubule.

The glomerulus is the filtration apparatus of the nephron. Defective filtration leads to development of glomerular disease as represented by nephrotic syndrome. In this part of thesis (chapter 3), in order to identify novel genes that are relevant to nephrotic syndrome, the zebrafish was first used as a screening system to isolate candidate genes from human datasets, including microarray data on kidney biopsies from human nephrotic patients. Rho-GTPase activating protein, IQGAP2, was identified as a candidate gene that is enriched in the glomeruli of the human kidney, downregulated in nephrotic patients. Subsequent functional analysis of IQGAP2 in the zebrafish revealed that disruption of IQGAP2 leads to a filtration defect of the glomerulus with effacement of podocyte foot processes, a characteristic pathological feature of nephrotic syndrome. These results suggest that downregulation of IQGAP2 may be potentially relevant to pathogenesis of nephrotic syndrome in humans. In addition, this study demonstrates the strength of the zebrafish model to achieve rapid assessments of gene function *in vivo*.

In the second part of thesis (chapter 4 & 5), we established the zebrafish as a system to investigate mechanisms underlying NaCl handling by the distal nephron. We particularly focused on sodium chloride cotransporter (NCC) expressed in the distal convoluted tubule in mammalian kidneys. NCC plays a critical role in NaCl reabsorption as evidenced by the monogenic disorders with pronounced alterations in blood pressure resulting from dysregulated NCC. The zebrafish possess the orthologue of NCC that is expressed in the pronephric distal late segment, corresponding to the mammalian distal convoluted tubule. We developed antibodies against total and phosphorylated forms of zebrafish NCC. These antibodies recognized presence of NCC in the pronephros and more importantly, phospho-antibodies could also detect changes in phosphorylation status of NCC after high salinity treatments of zebrafish. This

study shows that the zebrafish pronephros can be used to analyze activity of ion transporters and fundamental function of NCC in the distal tubule is likely conserved from teleosts to mammals. Furthermore, we generated transgenic zebrafish with mCherry expression in the distal segment using the promoter from *slc12a3* that encodes for NCC. This transgenic line should serve as a valuable tool for detailed analyses of function of the distal segment as well as for monitoring development of the distal nephron.

III ZUSAMMENFASSUNG

Die Niere ist für die Homöostase unseres inneren Milieus zuständig. In dieser Doktorarbeit wurde der Zebrafisch als Modellsystem zur Erforschung humaner Nierenfunktion-und Erkrankung genutzt. Dazu bietet das Zebrafisch-Modell ein umfangreiches Spektrum genetischer Werkzeuge. Der Pronephros des Zebrafisches ist der Erforschung leicht zugänglich, evolutionär hoch konserviert und mit dem Metanephros der Säugerniere vergleichbar. Die hier berichtete Forschung gliedert sich in zwei Teile; einem über den Glomerulus und einem über den distalen Tubulus.

Der Glomerulus ist der Filtrationsapparat des Nephrons. Ist er defekt, kann dies zu zahlreichen Erkrankungen führen, wie zum Beispiel dem nephrotischen Syndrom. In diesem Teil der Arbeit (Kapitel 3) wurde der Zebrafisch zunächst genutzt, um neue Genkandidaten zu identifizieren, welche relevant für die Entstehung des nephrotischen Syndroms sein könnten. Diese wurden mit humanen Nierenbiopsie-Datensätzen nephrotischer Patienten abgeglichen. Das Rho-GTPase aktivierende Protein IQGAP2 wurde als vielversprechender Kandidat identifiziert. Es ist in humanen Glomeruli stark exprimiert, jedoch in nephrotischen Patienten herabreguliert. In funktionellen Studien im Zebrafisch führte ein Ausschalten von IQGAP2 zu defekter Filtration mit einer Verschmelzung der Podozytenfussprozesse, einem klassischen Merkmal des nephrotischen Syndroms. Diese Ergebnisse deuten darauf hin, dass IQGAP2 potentiell relevant für die Pathogenese des nephrotischen Syndroms im Menschen ist. Weiterhin zeigt diese Studie eine Stärke des Zebrafischmodelles: die rapide Analyse von Genfunktionen *in vivo*.

Für den zweiten Teil der Arbeit (Kapitel 4 und 5) wurde der Zebrafisch als Modell zum Studium der Salzhomöostase im distalen Nephron etabliert. Dabei haben wir uns besonders auf den Natrium-Chlorid-Kotransporter (NCC) fokussiert, welcher im distalen Konvolut der Säugerniere exprimiert wird. NCC spielt eine wichtige Rolle bei der NaCl-Reabsorption, wie insbesondere in monogenetischen Erkrankungen mit dysreguliertem NCC zu sehen ist, die in Veränderungen des Blutdruckes münden. Zebrafische exprimieren ein zu NCC orthologes Protein im pronephrischen spät-distalen Segment, welches dem humanen distalen Nephron entspricht. Um dieses genauer zu studieren, entwickelten wir Antikörper gegen das NCC-Gesamtprotein und phosphorylierte Formen von NCC. Mit diesen Antikörpern konnten wir die

Präsenz von NCC im Pronephros nachweisen und insbesondere auch Veränderungen in der NCC-Phosphorylierung nach Hochsalzbehandlung zeigen. Diese Studie zeigt, dass der Pronephros des Zebrafisches dazu genutzt werden kann, Ionentransporteraktivität zu analysieren. Weiterhin scheint die Funktion von NCC im distalen Tubulus von Knochenfischen bis hin zu Säugetieren erhalten. Darüber hinaus haben wir transgene Fische mit mCherry-Expression im spät-distalen Segment unter der Kontrolle des Promotors für *slc12a3*, welcher NCC codiert, generiert. Diese transgenen Fische sind vielversprechend, um in Zukunft detailliert Funktion und Entwicklung des distalen Nephrons zu analysieren.

Chapter 1

General introduction

1.1. The kidney

The essential role of the kidney is to maintain homeostasis of the internal environment. This essential function is achieved by filtration of the blood plasma and subsequent modification of the filtrate to excrete as urine [1]. The functional unit of the kidney is the nephron. Each nephron is an independent entity and in mammals, about one million nephrons comprise one kidney (Fig. 1). A nephron consists of a glomerulus and a tubule. The glomerulus is a filtration apparatus of the nephron. The glomerulus is continuous with the tubular lumen through Bowman`s space. The tubule consists of epithelial cells subdivided into different segments with distinct functions in converting the filtrate into urine. The tubular segments include the proximal tubule, the thin loop of Henle and the distal tubule.

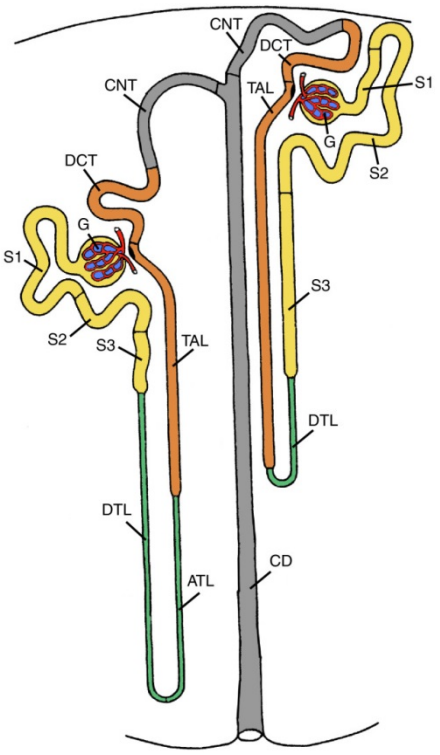

Fig. 1. Structure of the nephron. Superficial and juxtamedullary nephrons are depicted. G; Gloemrulus, S1, S2; Proximal convoluted tubule, S3; Proximal straight tubule, DTL; Descending thin limb, ATL; Ascending thin limb, TAL; Thick ascending limb, DCT; Distal convoluted tubule, CNT; Connecting tubule, CD; Collecting duct. Adapted from [2].

1.1.1. The glomerulus

The renal corpuscle is the site of the initial filtration of the blood plasma. The renal corpuscle consists of three components; the glomerulus, Bowman's space and Bowman's capsule (Fig. 2A). The glomerular filtration retains macromolecules larger than approximately 70 kDa in the blood while allowing passages of smaller molecules into the tubule. This size selectivity of the glomerulus is achieved by the glomerular filtration barrier (GFB), a three-layered structure composed of fenestrated endothelial cells (ECs), the glomerular basement membrane (GBM) and slit diaphragms (SDs) that bridges between podocyte foot processes (FPs) (Fig. 2B). The podocyte is a highly specialized epithelial cell that surrounds the glomerular capillaries (Fig. 2C) [3]. The podocyte cell body extends major and secondary processes that further extend FPs. The podocyte FPs form interdigitating structure with FPs from neighboring podocytes around the capillaries. Although endothelial cells and messangial cells also contribute to the GFB integrity, genetic studies of hereditary forms of glomerular disease explicitly points out that the podocyte plays a major role in the function of the GFB [4]. Damage to podocytes leads to filtration defects of the GFB, allowing leakage of macromolecules, clinically diagnosed as proteinuria. Nephrotic syndrome (NS) is a glomerular disorder characterized by severe proteinuria and edema [5]. A number of genes that are essential for the podocyte function are known to cause hereditary NS when mutated [6]. For instance, Nephrin is an essential structural component of podocyte SDs and mutations in the coding gene cause the most severe form of congenital NS [7]. The podocyte damage associated with glomerular disease is ultrastructurally characterized by effacement of FPs.

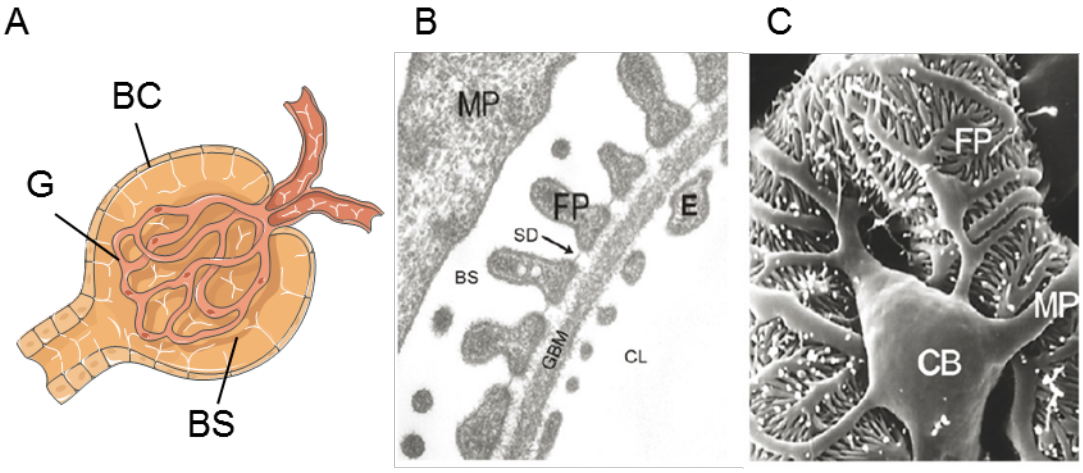

Fig. 2. (A) Schematic of the renal corpuscle. G; Glomerulus, BS; Bowman`s space, BC; Bowman`s capsule. (B) Transmission electron micrograph of the glomerular filtration barrier (GFB). Podocyte foot processes (FPs) are lining along the outer aspect of the glomerular basement membrane (GBM) with fenestrated endothelial cells (E) in the capillary side (CL). Each FP is bridged by slit diaphragms (SD) with adjacent FPs. MP; Major process. (C) Scanning electron micrograph of glomerular capillaries surrounded by podocyte FPs. (B)(C) adapted from [8].

1.1.2. The tubular system

The mammalian nephron tubule is largely divided into the proximal segment, the thin loop of Henle and the distal tubule [9]. The proximal segment is further divided into the proximal convoluted tubule (PCT) and the proximal straight tubule (PST). The typical morphological feature of the proximal tubular cells is infoldings of the apical membrane in the form of brush border. This amplified apical surface area of the proximal tubule reabsorbs a bulk of the filtrate to recover solutes and metabolite back into the circulation [10]. The thin loop of Henle is subdivided into descending (DTL) and ascending thin limbs (ATL). Although cells lining the ascending thin limb are impermeable to water, cells along the DTL are highly permeable and water is readily absorbed by osmotic gradient [11]. The distal tubule consists of the thick ascending limb of Henle's loop (TAL), distal convoluted tubule (DCT), the connecting tubule (CNT) and the collecting duct (CD). The distal tubule functions to fine-tune ion contents of the filtrate, such as Na^+, Cl^- and K^+ [12]. A number of solute transporters are expressed along the distal tubular cells. Genetic disturbances in genes encoding for these transporters cause monogenic disorders with pronounced alterations in blood pressure and ion balance, underscoring the importance of this tubule in maintenance of ion homeostasis [13].

1.2. The ontogeny of the kidney

Three different types of nephrons with increasing complexity are formed through the development of the mammalian kidneys; the pronephros, mesonephros and metanephros [14]. The first to form is the pronephros that arises from the intermediate mesoderm. At an early embryonic stage, the nephric duct appears and elongates caudally along the trunk to join with the urogenital sinus (Fig. 3A) [15]. The nephric duct induces the formation of tubules of the pronephros from the adjacent mesenchyme. The pronephros is a functional organ during development in lower vertebrates (e.g. fish, amphibian) [16]. In mammals, the pronephros is not functional and its main role is an induction of the mesonephric tubules in the adjacent nephrogenic cord [17]. As the anterior part of the nephric duct and pronephric tubules degenerate, mesonephric tubules are induced around the middle portion of the nephric cord [18]. The mesonephros is the final form of the kidney in lower vertebrates, but in mammals, it is a transient organ with a limited function before the formation of the metanephros [19]. The metanephros is generated by reciprocal interactions between the ureteric buds and metanephrogenic mesenchyme at the caudal end of the nephric duct [20]. The metanephrogenic mesenchyme causes branching of nephric ducts to induce elongation of the ureteric buds. Epithelial aggregates are formed around the tips of the ureteric bud. Each aggregated cluster first elongates to become a comma shape and then forms the “S-shaped body” (Fig. 3B). Then, epithelial cells start to differentiate into segment specific cell types, such as podocytes, proximal tubular cells and distal tubular cells. The branched ureteric bud eventually gives rise to the collecting ducts that become fused with the secretory nephrons derived from mesenchyme.

Similar cell types comprise every kidney form. Thus, not surprisingly, genes involved in the formation of one kidney type are similarly used for the formation of the other kidney types [21]. For example, the transcription factor, Pax2, regulates the development of the mesonephros and the metanephros. The mouse mutant for *Pax2* lacks the ureteric bud and the mesenchyme of the nephrogenic cord fail to epithelialize [22]. Moreover, Pax2 is required also for pronephric development. The zebrafish *pax2a* mutant, *no ithmus*, exhibits an abnormal pronephric tubular formation [23]. This transcription factor is evolutionarily well conserved and its orthogloue is already found in flies [24]. Therefore, although distinct in morphology, these observations suggest that there is a general principle underlying the development of each kidney type among vertebrates.

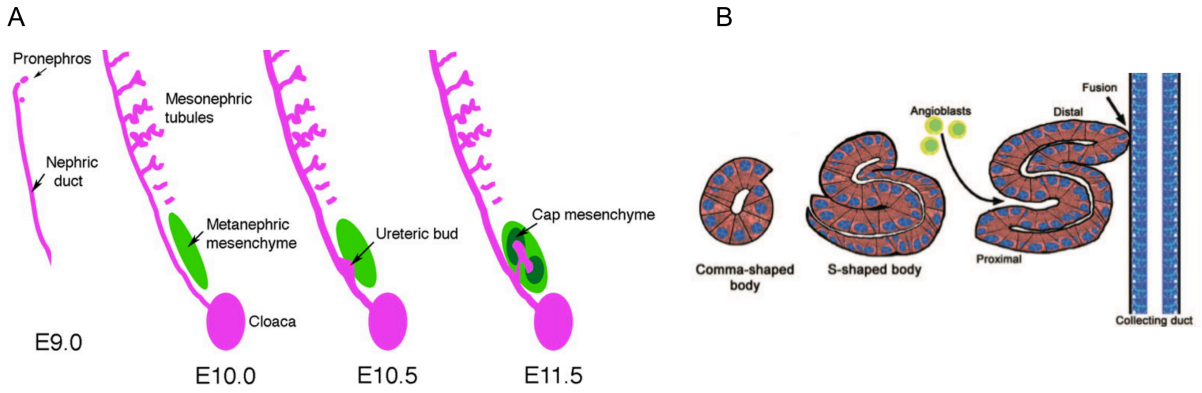

Fig. 3. Development of the mammalian metanephros. (A) Schematic drawing of the successive formation of three different types of nephrons during the mammalian kidney development. Adapted from [25]. (B) Maturation of a nephron through comma shape and S-shaped body of epithelial aggregates. Adapted from [15].

1.3. Animal models in renal research

Thanks to well-established gene manipulation techniques and similarity of the body plan to that of humans, the mouse model is still the major system to study human kidney disease. In mice, spatiotemporal control of gene targeting can precisely manipulate gene function for analyses of resulting phenotypes [26]. The mouse model will probably stay as a valuable model system in renal research, but investment of time and cost in using the rodent models meets with little success when it comes to rapid assessments of gene function. This is particularly a drawback since innovations of next-generation sequencing technologies now produce a large amount of comprehensive datasets, which necessitate high throughput functional analyses of candidate genes at a large-scale [27]. Due to advancements in genome editing technologies, the rat is becoming a good alternative rodent model to the mouse model for human diseases [28]. However, the use of this animal model is economically prohibitive and it is not a choice of animal for a large-scale genetic analyses.

Invertebrate models offer a high throughput genetic system with advanced tools of gene manipulation. Several work have demonstrated the use of *Drosophila* to study human kidney function [29]. The morphological and functional homology of *Drosophila* nephrocytes provides a system to study podocytes and proximal tubular cells of mammalian kidneys. The nematode, *Caenorhabditis elegans*, does not have a proper kidney, but it has been used to analyze cilia development and function [30]. As ciliary defect is a central factor in pathogenesis of

many of cystic kidney diseases, the nematode has potential to serve as a model system to study human kidney disease. A limitation of invertebrate systems resides in its simplicity that makes modeling complex human disorders often challenging.

With the conserved renal system as a vertebrate, *Xenopus* provides an informative system to study mammalian renal development and function [31]. The pronephric tubules of lower vertebrates were originally thought to be a simple duct without any different subdomains. However, studies in *Xenopus* pronephros revealed that the pronephric tubule in amphibian is actually divided into segments as in mammalian nephrons [2]. This led to a broad recognition that the pronephric tubules in lower vertebrates, including that of the zebrafish, consist of distinct subdomains likely with different functions [32]. Although informative in investigating the renal development and segmentation patterning, a limitation of the *Xenopus* model system is lack of genetic tools that allow robust gene manipulation. Moreover, its pronephros is not readily accessible for direct functional analyses of genes of interest. Also, the *Xenopus* model is not suited to large-scale genetic studies. Therefore, a vertebrate animal model that allows high throughput analyses of gene function with established genetic tools is desired.

1.4. The zebrafish model system: Another new wave

The zebrafish is a powerful vertebrate model to investigate human disease [33]. An array of powerful genetic tools is available in the zebrafish [34]. Most notably, functional knockdowns using morpholino oligonucleotides achieve rapid assessments of gene function. Of particular significance, a recent technological innovation in site-directed mutagenesis mediated by CRISPR/Cas9 system has begun to revolutionize reverse genetic studies in zebrafish [35]. CRISPR/Cas9 system uses components of a bacterial adaptive immune system and the strength of CRISPR/Cas9 is its simplicity [36]. It only requires a vector construction with a short stretch of target specific sequence downstream of the guide RNA element [37]. Simplicity of CRISPR/Cas9 system is by far above that of TALENs that were already surprisingly accessible compared to the original artificial endonuclease, zinc finger nucleases (ZFNs) [38]. With this technique, it is now possible to generate zebrafish mutants for genes of interest in a short period of time in any individual laboratories. In conjunction with the high fecundity and the optical clarity of embryos, the zebrafish serves as an accessible high throughput *in vivo* system to study biological processes that are relevant to questions of human disease.

One of the goals of application of the genome editing technology in zebrafish is to establish a technique to achieve a precise spatiotemporal control of gene manipulation in this animal model. In many cases, gene knockouts in a total animal induce embryonic lethality that prevents analyses of gene function at later stages. This is a significant limitation since many human diseases are adult onset. Moreover, even though animals are viable, when genes are expressed in several tissues and organs, total knockouts complicate the subsequent phenotyping by affecting multiple tissues and organs [39]. Therefore, confining gene disruption to only a specific tissue or cell type is required to analyze specific roles of genes in regions of interest. By driving Cas9 expression using a blood cell promoter from *gata1*, a recent work successfully disrupted the urod gene, encoding for an enzyme in heme biosynthesis, specifically in erythrocytes in zebrafish [40]. This work has opened a way for precise spatial control of gene manipulation in zebrafish.

Genome editing technologies, including CRISPR/Cas9 system, can be used also for site-directed knock-in approaches [41]. Coinjection of a donor vector containing a target specific sequence fused to a knock-in element induces double stranded breaks (DSBs) both in the genomic locus and the donor vector. The linearized vector integrates into the genomic locus in the process of the non-homologous end joining (NHEJ), thus achieving the insertion of exogenous DNA [42]. Furthermore, by constructing a donor vector with a knock-in element flanked by homology sequences to be targeted to a specific locus, a particular DNA element can be inserted when the DSB is repaired by homology directed repair [43]. This approach has been used to insert loxP sites, which promises us an important step toward conditional gene manipulation in the zebrafish [44]. Insertion of attP landing sites for PhiC31 integrase system should be of another importance in order to achieve site directed transgenesis [45]. These applications should inevitably lead to realization of precise modification of genomic loci as done in the mouse model via homologous recombination. By such approach, it would be possible to insert specific mutation sites or sites that modify function of protein products, for instance, such that proteins are constitutively active [46]. Application of CRISPR/Cas9 system to achieve gene replacement is now on our horizon [47]. Therefore, considering rapidity of functional analyses together with conservation as a vertebrate, the zebrafish is a powerful and versatile model system to analyze genetics of human disease.

1.7. The zebrafish pronephros

1.7.1. The glomerulus

As a highly accessible vertebrate model, the zebrafish pronephros has been increasingly used to study human kidney disease. The zebrafish pronephros consists of one single glomerulus and two bilateral tubules that extend along the body axis (Fig. 5A) [48]. The pronephros possesses a high degree of morphological and genetic conservation with mammalian metanephric nephrons. Cell types that are found in the mammalian nephrons also comprise the zebrafish pronephros [49]. Arising from the intermediate mesoderm, development of the pronephros progresses governed by pathways involving several transcription factors, such as pax2a, pax8 and lhx1a [50][51]. At 40 – 48 hpf, endothelial cells from the dorsal aorta invade into the glomerular epithelia and the glomerular filtration takes place. The GFB achieves the complete size selectivity approximately at 96 hpf, when the GFB acquires the typical three-layered structure with fully matured podocyte foot processes (Fig. 5B, C) [52].

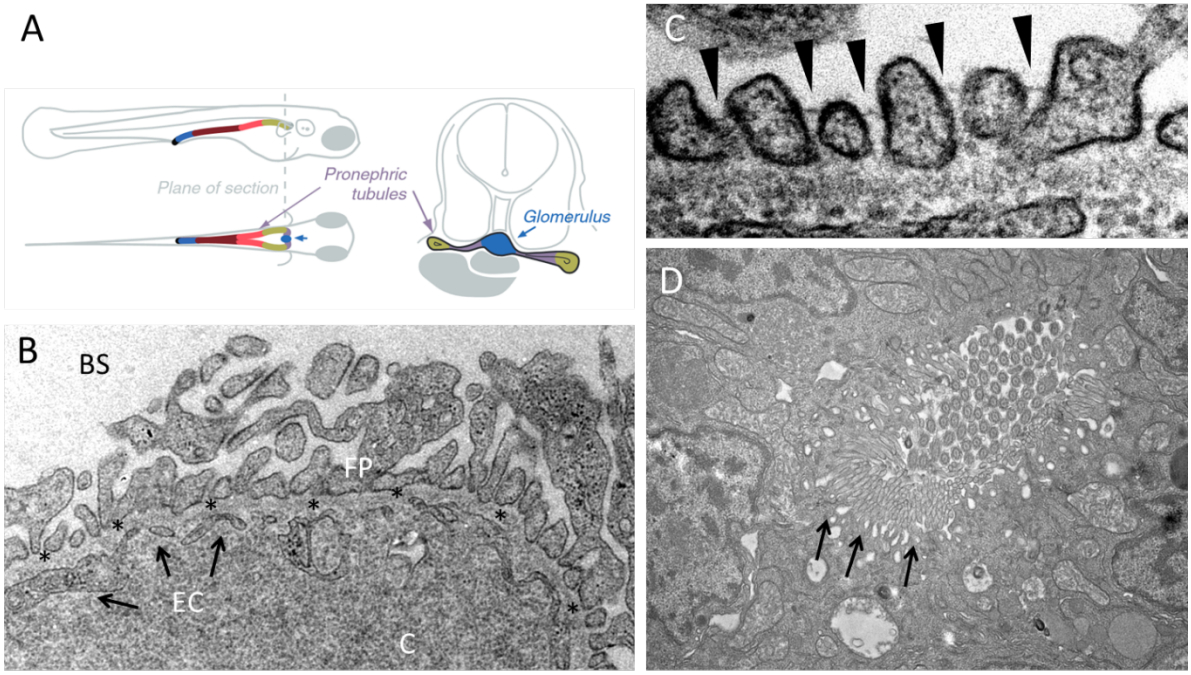

Fig. 4. (A) Schematic of the zebrafish pronephros. Adapted from [51]. (B) Electron micrograph of the glomerular filtration barrier in the pronephros. BS; Bowman`s space, FP; Podocyte foot processes, EC; Fenestrated endothelial cells, C; Capillary. Asterisks denote the glomerular basement membrane. (C) Higher magnification of podocyte FPs bridged by slit diaphragms (arrowheads). (D) Electron micrograph of proximal tubular cells in the pronephros. The brush border is evident on the apical membrane (arrows).

The zebrafish pronephric glomerulus is now frequently used to study genes implicated in human glomerular disease [53]. The glomerular filtration can be easily assessed by intravenous injection of fluorescent dextrans [54]. Genes encoding for critical components of the podocyte, including SDs and the actin cytoskeleton, are conserved in the zebrafish. For instance, the zebrafish possess the orthologues of Nephrin and Podocin. Functional knockdowns of *nephrin* and *podocin* lead to filtration defects associated with effacement of podocyte FPs, a characteristic pathological feature of human NS [52]. Fully exploiting the strength of transgenesis, a couple of useful transgenic zebrafish have been generated to facilitate studies of glomerular disease [55]. One is a zebrafish model of inducible podocyte injury using a podocyte specific promoter and nitroreductase (NTR) element. This transgenic line allows for analyses of podocyte injury through to adulthood by administraing a prodrug, metronidazole. The other one is a transgenic zebrafish that expresses vitamin D binding protein (VDBP) fused to GFP under a liver specific promoter. The size of GFP tagged VDBP is almost the same as mammalian albumin. Thus, in this fish line, VDBP-GFP is expressed in the liver and secreted into the circulation, mimicking the mammalian endogenous albumin. This transgenic zebrafish provides a reliable and robust system to analyze proteinuria resulting from podocyte injury.

1.7.2. The proximal tubule

The zebrafish pronephric tubule is subdivided into functionally different segments [56]. In fact, the segmented structure of the pronephric tubule is well conserved except for the thin limb, presumably because fresh water fishes do not need to reabsorb water in hypoosmotic environment. The pronephric proximal tubular cells possess micovilli on the apical surface, a typical feature of the mammalian proximal tubule (Fig. 5D). The proximal convoluted tubule (PCT) and proximal straight tubule (PST) express orthologous genes that are also expressed in the proximal tubules of the mammalian nephrons. For example, megalin (Low-density lipoprotein receptor-related protein 2; LRP2) mediates endocytosis in the proximal tubule, playing a critical role in recovery of metabolites [57]. Expression of the zebrafish orthologue of *megalin* is restricted to the PCT and its loss-of-function leads to a clearance defect of the pronephros, analogous to megalin deficiency in mammals [58]. Clinical significance of the proximal tubule is further seen in the case of acute kidney injury (AKI), where the proximal tubular cells are the major site of insult [59]. The nephrotoxic antibiotic, gentamicin, induces proximal tubular damage in zebrafish, recapitulating features of human AKI [60]. Therefore,

the zebrafish offers opportunities to study mechanisms involved in repair processes after AKI in humans [61]. In this respect, it is of particular note that lower vertebrates, including zebrafish, possess the capacity to regenerate nephrons *de novo* after injury [62][63]. A question of when and why during evolution this capacity was lost in mammals is still in a range of speculation, but studies of kidney injury using the zebrafish should also give us insights into renal regeneration, which may lead to development of a better therapeutic strategy to treat injured kidney in humans [64][65].

1.7.3. The distal tubule

The distal early (DE) and distal late (DL) correspond to the mammalian TAL and DCT, respectively. Osmoregulatory organs in teleost species are the gills, intestine and kidney. Mechanisms to keep ion homeostasis through the gills have been well characterized in the context of fish physiology [66]. In teleosts, chloride cells in the gills play a critical role in maintenance of ion compositions of body fluid, actively accumulating ions from the surrounding environment in fresh water while secreting excess ions in seawater [67]. In contrast to the gills, the contribution of the kidney in regulation of ion compositions of body fluid in teleosts has been less characterized. The distal tubular cells, however, are morphologically similar to the gill chloride cells, indicating that the distal nephron is also involved in regulation of ion contents of body fluid in teleosts [68]. Moreover, the zebrafish possess the orthologous genes for almost all the important ion transporters in the human kidney with conserved expression patterns in the distal segments of the pronephros [32]. This strongly suggests that the involvement of the distal nephron in regulations of ion balance is fundamentally conserved from zebrafish to humans. A recent work using the adult zebrafish reports differential regulations of genes encoding for some of these ion transporters in the kidney when fish were fed with Mg^{2+} deficient diet, presenting evidence that the zebrafish should be of use to study human hypomagnesemia and possibly other disorders with dysregulated ion homeostasis [69].

1.7.4. Use of the pronephros as a kidney disease model

Polycystic kidney disease (PKD) has been extensively studied using the zebrafish model. Because of its high frequency of occurrence, investigation of molecular mechanisms underlying pathogenesis of PKD represents an important field of research in nephrology [70]. PKD is

characterized by progressive cyst formation and development. Autosomal dominant PKD (ADPKD) is the most common form of PKD and in most cases, ADPKD is caused by mutations in two genes, polycystic kidney disease 1 (PKD1) and PKD2, encoding for Polycystin-1 and Polycystin-2, respectively [71]. In zebrafish, disruption of PKDs and other associated genes causes renal cyst formation, suggesting that pathways that lead to cyst formation in PKD are conserved [72][73]. Advantages of the zebrafish model can be fully exploited to investigate PKD. For instance, aberrant cellular Ca^{2+} signaling has been pointed out to play a role in cystogenesis in PKD [74]. Owing to innovations in transgenic approaches, zebrafish lines with a Ca^{2+} biosensor (e.g. GCaMP) achieve analyses of cellular Ca^{2+} signaling *in vivo* [75]. This should greatly facilitate research into involvement of Ca^{2+} signaling in pathogenesis of PKD. Furthermore, using signaling responsive elements, the zebrafish transgenesis also offers signaling pathway reporter lines [76]. Several important signaling pathways, such as Wnt and cAMP, are known to be involved in pathogenesis of PKD [77][78]. Direct visualization of these pathways in living animal using such transgenic zebrafish should further help us deepen our understanding of pathogenesis of PKD pathogenesis. These approaches are applicable to studies of other renal diseases and hence, the zebrafish is about to break a new ground in renal research.

1.8. Aims of the thesis projects

The overall aim of this thesis is to utilize the zebrafish model to investigate molecular mechanisms underlying renal function, of which disruption leads to disease pathogenesis in humans. Chapter 3 describes the use of the zebrafish pronephric glomerulus as a genetic system to isolate and analyze novel genes that may be relevant to human NS. Although characterization of single-gene causing NS have contributed to our understanding of podocyte biology, detailed mechanisms that maintain the podocyte integrity remains to be clarified. Thus, adding a new genetic factor to our knowledge on the podocyte should be of significance. Chapter 4 and 5 report the exploration of the use of the zebrafish pronephric tubule to study regulations of NaCl balance via the thiazide-sensitive NaCl cotransporter (NCC) in the distal kidney. In order to establish the zebrafish pronephros for analyses of NCC, we generated two tools; antibodies that recognize total and phosphorylated forms of the zebrafish orthologue of NCC, and a distal nephron specific transgenic zebrafish using the promoter from *slc12a3*. With these tools, we present a proof-of-concept to use the zebrafish for analyses of tubular function and disorders.

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Chapter 2

Reverse genetics tools in zebrafish: A forward dive into endocrinology

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Article published in *General and Comparative Endocrinology*

Personal contribution:

Preparation of all figures and writing of the manuscript

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Communication in Genomics and Proteomics

Reverse genetics tools in zebrafish: A forward dive into endocrinology

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Zebrafish
Reverse genetics
Transgenesis

ABSTRACT

The zebrafish is a powerful genetic model organism. In recent years, zebrafish has been increasingly used to model human diseases. Due to a number of recent technological advancements, the genetic tool box is now also stocked with sophisticated transgenic and reverse genetic tools. Here, we focus on both commonly used and recently established reverse genetic and transgenic tools available in zebrafish. These new developments make the zebrafish an even more attractive animal model in comparative endocrinology.

1. Introduction

The last three decades have seen the establishment of the zebrafish as a major vertebrate model organism for studies of human diseases. The zebrafish made its first splash as a model for genetic control of vertebrate development due to its favorable biological characteristics such as high fecundity, transparency and external development of embryos (Streisinger et al., 1981). Zebrafish genetics entered the picture and gained prominence with the two first large-scale mutagenesis screens performed in any vertebrate species (Driever et al., 1996; Haffter et al., 1996). At this time in the 1990s and in the following years, zebrafish genetics was nearly exclusively confined to forward genetic studies, exploiting the huge number of available mutant strains, many of which proofed to be relevant for our understanding of mechanisms of pathogenesis of a number of human diseases (Fishman Genomics, 2001; Lieschke and Currie, 2007). Genetic studies of human disorders benefit from the investigation of specific target genes of interest. In contrast to forward genetics, the lack of tools to manipulate specific genes of interest hindered the realization of the full potential of the zebrafish for modeling human diseases (Skromne and Prince, 2008). However, recent technological advances, mainly in antisense morpholino mediated gene knockdown, transgenics using the Tol2 system and the induction of heritable genetic alterations with zinc finger nucleases, TALENs and TILLING technology heralds a new era in zebrafish reverse genetics.

The strengths of zebrafish have been largely realized in the study of developmental biology, as is also the case for endocrine systems in zebrafish (Lohr and Hammerschmidt, 2011). Studies on zebrafish endocrine systems have revealed that the endocrine systems between humans and zebrafish are remarkably well conserved (McGonnell and Fowkes, 2006). Significantly, studies on zebrafish endocrine systems show that the development of endocrine systems mostly complete within 5 days post fertilization (dpf) in zebrafish. In other words, it is possible to model the human endocrine systems in developing zebrafish, taking full advantage of the strengths of this animal model.

In this review, we discuss several reverse genetics approaches that have been established in zebrafish as well as emerging techniques for targeted gene knockout including TALENs, and their application for studies of human endocrine systems.

2. Reverse genetic approaches in zebrafish

The mouse model has been a dominant system to study disease related genes because of conservation of gross anatomy with humans and sophisticated gene manipulation techniques made possible by homologous recombination (Thomas and Capecchi, 1987). Although application of gene targeting by homologous recombination has been explored in zebrafish, these attempts have only met with little success, mainly due to the absence of proper embryonic stem cells (Sun et al., 1995). However, other methods to manipulate gene function have gained prominence in the zebrafish. Morpholino antisense oligonucleotides and TILLING are two main reverse genetics methods, and now artificial endonuclease en-

Abbreviations: TALEN, transcription activator-like effector nucleases; ZFN, zinc finger nucleases; TILLING, targeted induced local lesions in genomes; UAS, upstream activating sequence.

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http://dx.doi.org/10.1016/j.ygcen.2013.02.002

zymes, ZFNs and TALENs, are emerging as a new technique to study specific genes of interest.

2.1. Morpholino knockdown

RNAi mediated gene knockdown technique is widely used in a variety of model systems from cell cultures to mammalian systems. Although there have been a few successful efforts to apply this technique in the zebrafish, RNAi mediated gene knockdown is not widely applicable in the zebrafish (Kelly and Hurlstone, 2011; Oates et al., 2000). Instead, knockdown of gene function by morpholino antisense nucleotides is the most widely used reverse genetics technique to study genes of interest in the zebrafish (Bedell et al., 2011). Morpholinos are chemically synthesized nucleotides with morpholine rings, thereby resistant to breakdown by nucleases (Nasevicius and Ekker, 2000). Usually morpholino sequences are designed to bind in the vicinity of the start codon to block initiation of translation or to splice acceptor sequences to cause abberantly spliced mRNAs.

Morpholinos are injected into freshly fertilized eggs and effectively block mRNA translation or splicing of target genes up to 5dpf, before becoming too diluted to efficiently interfere with translation. At this stage of development, most organs, including endocrines systems, are fully functional in zebrafish (Lohr and Hammerschmidt, 2011). The biggest advantage of morpholinos is their ease of use and quick read-out. Together with the high fecundity of zebrafish, morpholinos can be injected into several hundreds of embryos in one experiment and resulting phenotypes are readily observed in those embryos a few days later (Fig. 1). Furthermore, co-injection of a combination of up to three morpholinos can be performed to achieve double and triple knockdowns of genes of interest (McNulty et al., 2005). In addition, concentrations of morpholinos injected can be titrated in order to investigate dose-dependent resulting phenotypes. One exemplary application to the field of endocrinology is the downregulation of the zebrafish *irx3a* orthologue (Ragvin et al., 2010). This transcriptional regulator is expressed in the kidney, hypothalamus and endoderm derived tissues. Morpholino-based downregulation of *irx3a* increased the mRNA expression level of ghrelin while decreased that of insulin, demonstrating an involvement of irx3a in the regulation of - and -cells of the pancreas.

Though highly efficient, knockdown experiments using morpholinos require optimization of the injection dose and careful observation of resulting phenotypes as there is a potential risk of off-target effects, which may cause unspecific phenotypes (Eisen and Smith, 2008). Also, the effectiveness of the downregulation needs to be carefully assessed, e.g. by demonstrating the reduction or absence of the target protein in western blots. Keeping these experimental precautions in mind, morpholino-based knockdown is one of the most advantageous techniques the zebrafish has to offer. In addition to the conventional knockdown of translation of target mRNAs, morpholinos can also be used to block the maturation of microRNAs (miRNAs) as well as to inhibit their binding to the target mRNAs, facilitating assessment of target genes of miRNAs (Staton and Giraldez, 2011).

2.2. TILLING

TILLING (targeted induced local lesions in genomes) is a combined method of forward and reverse genetics based on chemical mutagenesis to isolate mutants harboring point mutations in genes of interest (Wienholds et al., 2003). Firstly, male fish are mutagenized by ENU (N-Nitroso-N-ethylurea), a chemical mutagen, and subsequently, mutations in target genes are sought by sequencing the target regions from genomes extracted from the mutagenized individuals (Fig. 2A). Although TILLING is a technically efficient method to obtain fish with mutations in genes of interest, since it requires a large-scale sequencing of a large pool of individual fish, it is difficult to conduct as a routine technique in most individual laboratories. Therefore, community-based zebrafish mutation projects by TILLING were established by several consortia (e.g. Sanger Institute) and the information and resources are shared via the zebrafish community database (http://zfin.org/).

2.3. ZFNs

Zinc finger nucleases (ZFNs) are artificial endonuclease enzymes originally introduced as a hybrid restriction enzyme (Kim et al., 1996). ZFNs consist of two domains. One is the zinc finger motif domain that recognizes a specific sequence of genomic DNA and the other is the *Fok*I restriction enzyme domain that cuts double-stranded DNA (Ekker, 2008). A DNA double-stranded break

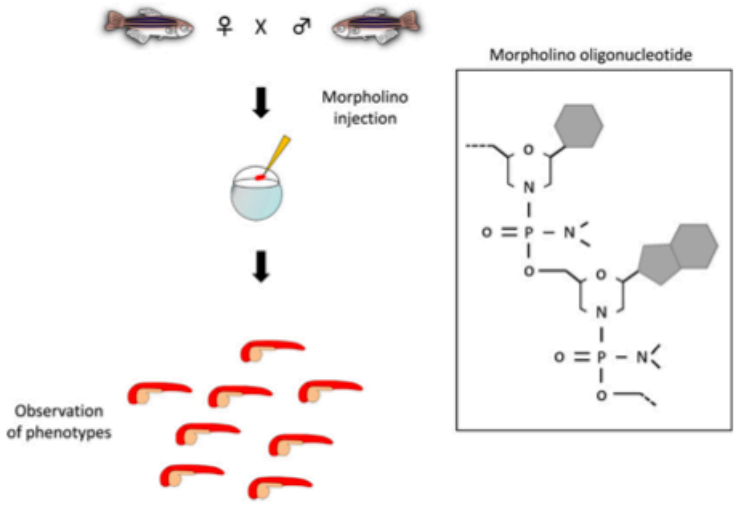

Fig. 1. Overview of a knockdown experiment by morpholino antisense nucleotides. A chemical structure of a morpholino is shown. Morpholinos are normally injected into fertilized embryos at the one cell stage. Effects of knockdowns of target genes are readily observed in these embryos up to 5dpf.

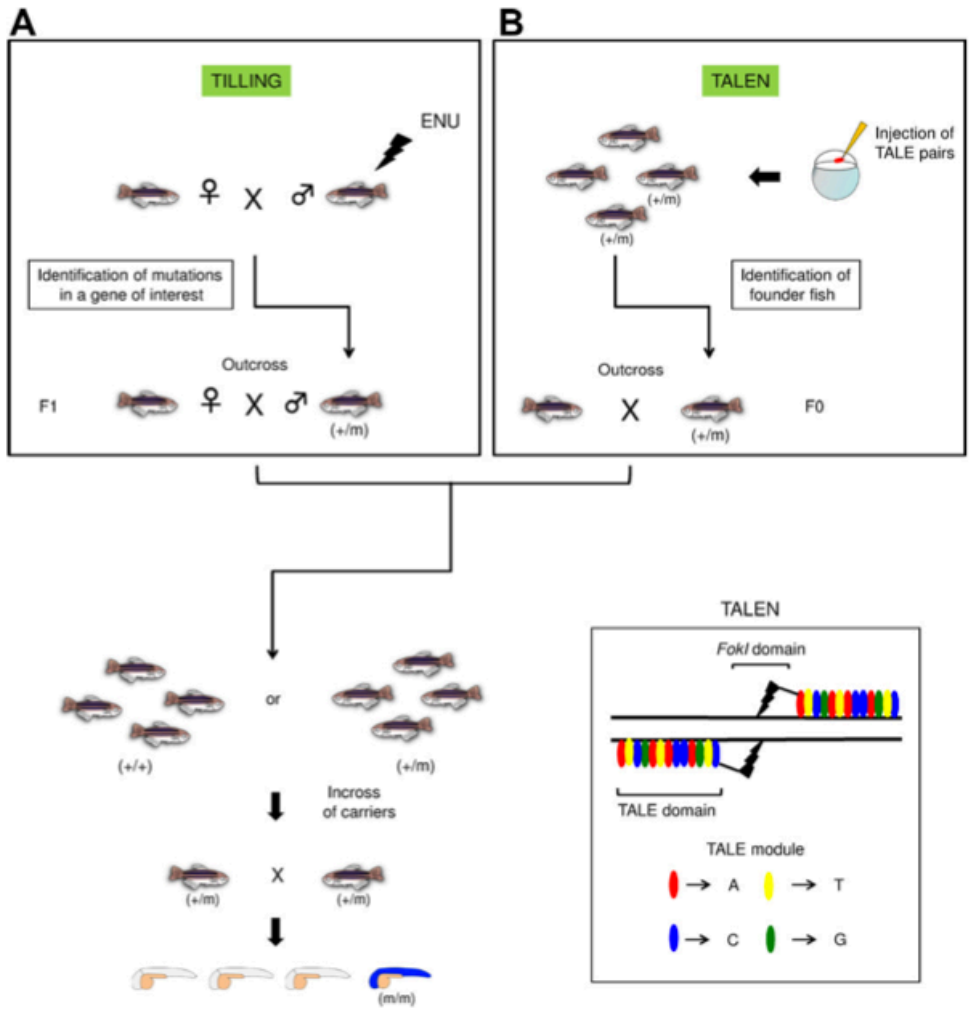

Fig. 2. Schematic representation of procedures to obtain knockout zebrafish by TILLING and TALENs. (A) In a conventional TILLING approach, as a first step, male fish are mutagenized by ENU and outcrossed to obtain the F1 founder generation. The resulting F1 fish are screened (e.g. by PCR based techniques and sequencing) for individuals with mutations in genes of interest. The mutation carriers are then crossed with wild type female fish to obtain the F2 generation. Incrossing of the F2 families results in generation of the F3 fish with homozygous mutations in target genes. "m" indicates a mutation in genes of interest. (B) In a TALEN approach, gene specific TALENs are injected into fertilized embryos at the one to two cell stage. These embryos are raised to adulthood and individual fish carrying mutations (founder fish) are screened. The founder fish are then crossed with wild type fish to obtain the F1 generation. Incrossing of the F1 families results in the F2 families that include fish that are homozygous for mutations in target genes. Of note, it is one generation shorter to obtain homozygote fish in the TALEN approach than the TILLING approach. A schematic of a TALEN is also shown. Four colored ovals represent each TALE module of the DNA binding domain of TALENs that recognizes a specific base.

is repaired by homologous recombination or a non-homologous end joining (NHEJ). As NHEJ is an error-prone process, it creates small deletion/insertion ('indel') mutations in a site of lesion. Thus, by engineering the zinc finger domains to bind specific loci of genomes, it is possible to introduce mutations into genes of interest.

ZFNs are injected into fertilized eggs at the one to two cell stages. Individuals harboring mutations in a target gene are subsequently identified and these founder fish are outcrossed to obtain an F1 generation. After confirming the mutations in the F1 generation, they are incrossed and the resulting F2 generation with a homozygous mutation in a gene of interest can be subject to analysis of the gene function. Although genes in the endocrine systems of zebrafish have not yet been targeted by ZFNs, there is a growing list of zebrafish lines generated by this technology (Lawson and Wolfe, 2011).

Targeted mutagenesis by ZFNs is simple in theory and ZFNs are indeed a viable tool for targeted mutagenesis, but the production of validated ZFN pairs is technically challenging and costly. In addition, ZFNs have a potential risk to cause off-target effects, which may yield unspecific phenotype unrelated to the function of target genes (Pattanayak et al., 2011). One of the disadvantages of ZFNs is their complexity regarding the designing of DNA binding domains (Zhu et al., 2011). ZFN binding domain contains 3 to 4 DNA recognition modules, each of which recognizes 3 DNA bases. However, when all the modules are assembled as one DNA binding domain, the specificity of DNA binding can decrease and therefore, designing specific ZFN pairs that recognize specific loci of the genome is selective, making some genes unavailable for targeted knockout via ZFN.

2.4. TALENs

Based on the same principal as ZFNs, the transcription activator-like effector nucleases (TALENs) are emerging as a new and

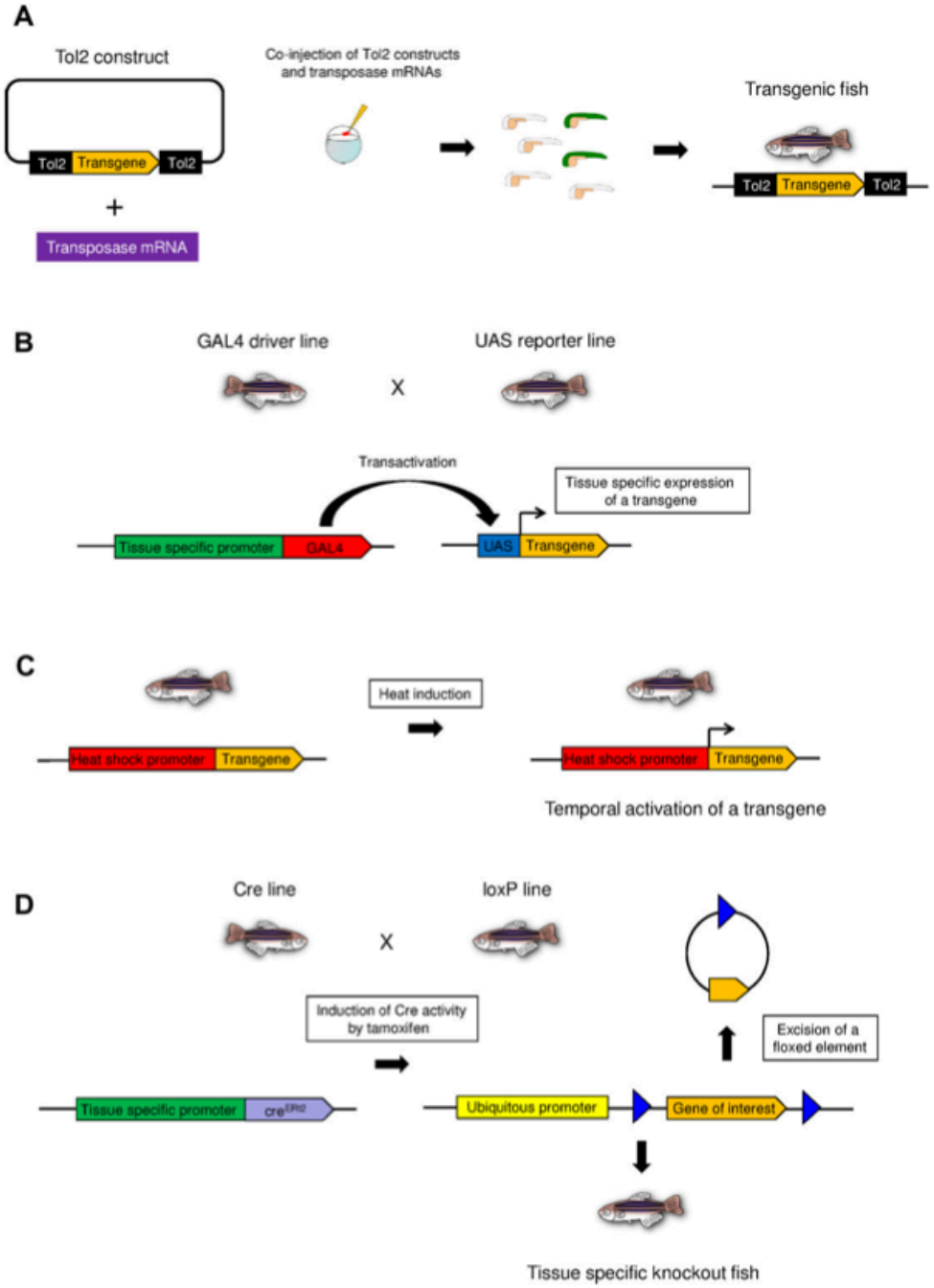

Fig. 3. Schemes for transgenic approaches. (A) Tol2 mediated transgenesis. Tol2 expression constructs contain transgenes flanked by the Tol2 transposable elements. Co-injection of the Tol2 constructs with mRNAs for transposase greatly enhances efficiency of integration of transgenes. (B) GAL4/UAS system. Upon crossing of a GAL4 driver line and UAS effector line, expression of GAL4 driven by a tissue specific promoter transactivates expression of a transgene through the UAS effector element in the resulting embryos. (C) Heat induced expression of a transgene. The heat shock promoter drives expression of a downstream transgene upon rise of the temperature. (D) Conditional knockout via the inducible Cre/loxP system. The Cre activity induced upon administration of tamoxifen excises a gene of interest flanked by loxP sites expressed under the ubiquitous promoter. By using a tissue specific promoter upstream of cre^{ER2}, conditional tissue specific knockouts can be achieved. Blue triangles indicate the loxP element.

efficient tool for targeted mutagenesis (Clark et al., 2011). TALENs use the same functional domains, namely the restriction enzyme domain and the DNA binding domain. In the case of TALENs, the DNA binding domain is derived from plant pathogenic bacteria with TALE modules recognizing specific bases (Fig. 2B) (Cade et al., 2012). TALENs are far more flexible and much simpler to generate than ZFNs because each TALE module of the DNA recognition domain of TALENs consists of a cipher for one particular DNA base and can be easily assembled one by one. Although off-target effects of TALENs still need to be evaluated with more accumulating expe-

rience, TALENs are increasingly used as an easily accessible gene targeting technique for many laboratories.

3. Transgenic approaches

Tissue specific expression of a transgene in in vivo system is a powerful tool for genetic studies. Transgenesis was historically the weakness of the zebrafish model system, but owing to extensive efforts to improve transgenesis in zebrafish, it turned into one of the most appealing features of zebrafish. There are several ways to enhance transgenesis in zebrafish, such as co-injection of *I-SceI* meganuclease, but the most common method currently largely in use is utilization of the Tol2 transposon system (Grabher and Wittbrodt, 2008; Kawakami, 2004). The Tol2 transposable elements were originally discovered in the Japanese medaka *Oryzias latipes* (Kawakami et al., 2000). Co-injection of expression constructs containing transgenes flanked by the Tol2 elements with the transposase mRNAs greatly enhances the efficiency of transgenesis in zebrafish, including germ line transmission (Fig. 3A). Together with the development of the gateway technology that significantly facilitates flexible assemblies of expression constructs, researchers can now fully exploit advantages of the zebrafish as a model system (Kwan et al., 2007).

3.1. GAL4/UAS

The GAL4/UAS system is a standard approach in Drosophila (Duffy, 2002). Since the improvement of the transgenesis in zebrafish by the Tol2 system, use of the GAL4/UAS system has been becoming a more and more common approach in zebrafish (Scheer and Campos-Ortega, 1999; Scott, 2009). By fusing a transgene of interest under the control of the UAS promoter, the GAL4/UAS system allows tissue specific expression of the transgene when combined with the tissue specific GAL4 driver line (Fig. 3B). Using this technology, overexpression of a gene of interest with tissue specificity can be achieved. The most advantageous feature of the GAL4/UAS system is that an array of GAL4 driver lines can be combined with a range of UAS effector lines, allowing a multitude of spatiotemporal regulation of gene functions. The stable UAS transgenic zebrafish lines generated so far express fluorophores in GAL4 positive cells to visualize gene function and cell specific behaviors. Future work is expected to produce various UAS effector lines that activate or silence gene function under an array of multiple GAL4 driver lines (Muto and Kawakami, 2011).

3.2. Heat shock promoters

Heat shock promoters drive expression of transgenes when the surrounding temperature is raised to 38 °C, allowing temporal and spatial control of transgene expression (Fig. 3C) (Halloran et al., 2000). This technique is a powerful tool when studying effects of overexpression of genes of interest at different time points during the development. In addition, being a small water-living animal, the zebrafish is a particularly viable model system for this technique as the surrounding temperature is easily controlled (Shoji and Sato-Maeda, 2008). One issue entailing the use of the heat shock promoters is a leakiness of the promoter activity, resulting in the basal expression of the downstream genes even at the permissive temperature (Hans et al., 2009). However, this leakiness can be circumvented by integrating this technique in the ligand-inducible Cre/loxP system (Hans et al., 2011). Furthermore, a method using a modified soldering iron has been developed to enable a site-specific heat induction in order to achieve spatial specificity of transgene control as well as temporal specificity (Hardy et al., 2007; Placinta et al., 2009).

3.3. Cre/loxP system

One of the remaining challenges in zebrafish reverse genetics is to accomplish a conditional knockout/down technique (Fig. 3D). In that respect, the mouse model is still the most sophisticated model system due to the well-established Cre/loxP system, by which mouse geneticists can manipulate genes of interest in a spatiotemporal manner at will (Branda and Dymecki, 2004). It is a natural course of progress to apply the Cre/loxP system to the zebrafish model system to achieve the spatiotemporal control of gene expression, but shortage of a promoter/enhancer that ubiquitously drive expression of transgenes has been an obstacle (Higashijima et al., 1997; Yoshikawa et al., 2008). Although several elements that are assumed to have broad control of genes have been identified, including *h2afx*, *tbp* and *-actin* promoter, these expression driving elements are inactivated during the development and thus cannot be a proper ubiquitous promoter (Burket et al., 2008).

Recently, the promoter of the zebrafish *ubiquitin*, a ubiquitin peptide precursor, that is highly conserved from plants to humans, was isolated and shown to drive expression of transgenes at all stages of the zebrafish development and in all adult organs (Mosimann et al., 2011). Subsequently, stable EGFP transgenic lines were generated using the *ubiquitin* promoter that showed strong ubiquitous expression of EGFP from an early stage of development to adulthood. Furthermore, the same group created *ubi:cre*^{ERt2} transgenic lines and detected Cre activity at various time points of development upon administration of tamoxifen. By crossing this *ubi:cre*^{ERt2} lines with *ubi* reporter transgenics flanked by two loxP sites, a strong Cre mediated reporter activity was successfully demonstrated.

Although the *ubi* promoter activity reported in this study varies depending on cell types in different tissues and loxP reporter activity upon Cre exposure has positional effects, isolation of a ubiquitous promoter and application of the Cre/loxP system in zebrafish has demonstrated the potential of this animal model to become a more sophisticated and flexible system in the near future.

3.4. Targeted cell ablation

Cell type specific ablation is an important tool for regeneration studies and enhanced transgenesis made targeted cell ablation one of the powerful methods in zebrafish. Bacterial nitroreductase (NTR) converts metronidazole (Met) into a cytotoxic agent (Curado et al., 2008). By expressing NTR under a promoter that drives expression in a cell type specific manner, cell populations of interest can be ablated in a time point specific manner upon administration of Met. One example of the application of this technique is that insulin producing -cells were successfully ablated in the pancreas, resulting in reduced mass of -cells (Pisharath et al., 2007). This technique can be combined with the GAL4/UAS system, where NTR is expressed under the UAS promoter, which is transactivated by the GAL4 driver in a particular cell type (Gray et al., 2011). With this combination, targeted cell ablation can be performed with more flexibility.

4. Conclusion

The zebrafish offers a diverse range of approaches to study human diseases. Recent advancements in reverse genetics technologies further accelerate the use of the zebrafish as a model system. Many of the key factors in endocrine systems are conserved from humans to zebrafish and therefore, considering the useful biological features, together with reverse genetics techniques in hand, the zebrafish should attract more attention as a major organism to study endocrinology. Endocrinology research

using zebrafish has the potential to provide more insights into our understanding of human endocrine systems and contribute to elucidations of genetic mechanisms of human diseases caused by disruptions of the endocrine systems.

Acknowledgments

We apologize in advance to all the researchers whose work we could not cite due to space limitations. Work in the authors' lab is supported by the Swiss National Science Foundation (31003A_135598), EU ZF-HEALTH 7th frame work program and the Zurich Center for Integrative Human Physiology.

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Chapter 3

IQGAP2 is a conserved podocyte gene required for the glomerular filtration barrier in zebrafish

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Manuscript accepted for publication to *Kidney International*

Personal contribution:

Cloning, *in situ* hybridization, morpholino knockdowns, histology, antibody development, immunohistochemistry, filtration assay, electron microscopic analysis, preparations of all figures and writing of the manuscript except for those for human dataset analysis in materials and methods and result sections.

3.1. Abstract

Podocytes are critical for the maintenance of the glomerular filtration barrier. Podocyte dysfunction impairs the size selectivity of the glomerular filter, leading to proteinuria, hypoalbuminuria and edema, clinically defined as nephrotic syndrome (NS). Hereditary forms of NS are linked to mutations in podocyte-specific genes. To identify gene products possibly contributing to podocyte dysfunction in acquired NS, we analyzed human glomerular gene expression datasets for glomerulus-enriched gene transcripts differentially regulated between control samples and samples isolated from patients with NS. Candidate genes were screened by *in situ* hybridization for expression in the zebrafish pronephros, an easy-to-use *in vivo* assay system to assess podocyte function. One of the identified glomerulus-enriched products was the Rho-GTPase binding protein, IQGAP2. Immunohistochemistry revealed a strong abundance of IQGAP2 in normal human and zebrafish podocytes. In zebrafish larvae, morpholino-based knockdown of *iqgap2* caused a mild foot process effacement of zebrafish podocytes and a cystic dilation of the urinary space of Bowman's capsule upon the onset of urinary filtration. Moreover, the glomerulus of zebrafish morphants, but not of controls, showed a glomerular permeability for injected high molecular weight dextrans (≥ 500 kDa), indicating an impaired size selectivity of the glomerular filter. Thus, IQGAP2 is a Rho-GTPase binding protein that is highly abundant in human and zebrafish podocytes, which controls normal podocyte structure and function as evidenced in the zebrafish pronephros.

3.2. Introduction

Nephrotic syndrome (NS) is a glomerular disorder characterized by proteinuria, hypoalbuminemia, dyslipidemia and edema. In NS, an impaired glomerular filter leads to leakage of macromolecules from the blood into the urine, explaining the severe proteinuria. In the healthy glomerulus, the size selectivity of the glomerular filter is achieved by the glomerular filtration barrier (GFB), a three-layered structure consisting of fenestrated endothelial cells (EC), the glomerular basement membrane (GBM) and podocyte's slit diaphragms (SD) [1]. The podocyte is a highly specialized and motile epithelial cell surrounding glomerular capillaries. The foot processes (FPs) of adjacent podocytes interdigitate and form filtration slits that are bridged by SDs. Mutations in genes encoding for structural components of the SD (e.g. NPHS1, NPHS2 and CD2AP) as well as proteins associated with the podocyte actin cytoskeleton (e.g. ACTN4) cause hereditary NS [2][3][4]. Although positional cloning of mutated loci in human patients identified a number of genes that are critical for the pathogenesis of hereditary forms of NS, we know very little about the molecular mechanisms that lead to non-hereditary, acquired forms of NS [5]. In this context, it is interesting to note that certain forms of acquired NS (e.g. minimal change disease) respond well to the treatment of glucocorticoids (GCs) [6][7]. Given that the beneficial effect of GCs is thought to be independent from their anti-inflammatory role and podocytes express the glucocorticoid receptor (GR)[8], which acts as a ligand-dependent transcription factor, it is tempting to speculate that the GCs may directly act on the podocyte and restore a potentially dysregulated transcriptional program involved in the pathogenesis of steroid-sensitive NS.

In this study, we aimed to identify putative gene products dysregulated in patients with NS contributing to the pathogenesis of acquired NS. To reach this end, we screened human datasets from the European renal cDNA bank for glomerulus-enriched candidate gene transcripts that were differentially expressed in kidney biopsies from normal humans and patients with steroid-sensitive NS. To analyze the functional role of candidate genes, we then took advantage of the zebrafish model, in which the pronephros of the larvae offers a system that is easily accessible for genetic manipulations and morphological and functional analyses [9]. Previous studies demonstrated the strength of this model system for the analyses of the functional role of genes causing hereditary forms of glomerulopathies and cystic kidney diseases [10][11], we now aimed to use the system to characterize the role of podocyte genes identified from the human datasets mentioned above. In our screens, we identified IQGAP2, the IQ motif containing GTPase activating protein, as a potential candidate gene. Subsequent *in situ* hybridization confirmed expression of the zebrafish orthologue of IQGAP2 in podocytes in the pronephric glomerulus. Gene knockdown of *iqgap2* in zebrafish caused a progressive effacement of podocyte FPs and an impaired glomerular filtration, suggesting that IQGAP2 is important for the maintenance of the podocyte FP architecture in the functional GFB.

3.3. Materials and methods

Glomerular mRNA analysis in human kidney biopsies

Human renal biopsy specimens were collected in an international multicenter study, the European Renal cDNA Bank-Kröner-Fresenius biopsy bank [ERCB-KFB (http://www.research-projects.uzh.ch/p9291.htm)] [12]. Biopsies were collected after informed written consent was obtained and with approval of the local ethics committees. The tissue was transferred to RNase inhibitor immediately after the biopsy was taken and microdissected into glomerular and tubulointerstitial compartments. Total RNA was isolated from microdissected glomeruli and reverse-transcribed as previously described [13].

Gene-expression profiling from glomeruli of patients with steroid-sensitive nephropathies such as focal-segmental glomerulosclerosis (FSGS, n = 19), membranous nephropathy (MGN, n = 21), and minimal change disease (MCD, n = 5), as well as pretransplant biopsies from living renal allograft donors as controls (n = 32) was performed in two independent hybridization experiments using the Affymetrix HG-U133A and HG-U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA) [13]. For microarray analysis a single probe-based analysis tool, ChipInspector (Genomatix Software GmbH) was used for transcript annotation, total intensity normalization, significance analysis of microarrays, and transcript identification based on significantly changed probe signals [13]. Only probes that match uniquely to the genome and to at least 1 transcript were retained for further analysis. The statistic algorithm in ChipInspector is a T-test with a permutated artificial background based on [14]. It enhances the original significance analysis of microarrays (SAM analysis) algorithm. The input data for the SAM analysis were single probe values, and the resulting probes with significant alteration of signal levels were subsequently matched with the corresponding transcripts (which may be more than 1 per gene). For each probe, a score on the basis of its fold change relative to the standard deviation of repeated measurements for this probe was calculated. Probes with scores higher than a certain cutoff (positive and negative delta value) were deemed significant. In addition, the false discovery rate (FDR) for each delta threshold was calculated (positive and negative FDR). The ChipInspector algorithm independently calculates these numbers for the sets of positive and negative data point (Supplementary Table 1). No fold change cut-off was applied, but minimum coverage of probes with significant alteration per transcripts was set as 3.

Immunohistochemistry

2 µm sections from formalin-fixed and paraffin-embedded specimens from allograft biopsies were subject for immunohistochemistry. Antigen retrieval and immunohistochemistry were performed as

previously described using a polyclonal rabbit anti IQGAP2 antibody (Cell Signaling Technology, Bio-Concept, Allschwil, Switzerland) [15]. Archival renal tissues were used with approval of the local ethics committee.

Zebrafish strains and husbandry

Zebrafish were maintained under a 14h/10h light/dark cycle. Fish were bred as previously described and the embryos were raised at 28 ^{0}C in E3 medium [16]. *Tg(wt1b:*EGFP*)* line was a kind gift from Dr. Christoph Englert (Leibniz Age Research, Jena, Germany). All experiments were performed according to the European Communities Council Directive for animal use in science (2010/63/EU) and in accordance with Swiss laws.

Isolation of iqgap2

Zebrafish cDNAs were synthesized from total RNA from 5 dpf larvae using Oligo dT primers with SuperScriptIII Reverse Transcriptase (Invitrogen, Basel, Switzerland). The zebrafish iqgap2 cDNA fragment was amplified by PCR from the cDNAs using the following primer pair: (5'- TGTACCATGAAGACGGAG-3' and 5'- TGTACCATGAAGACGGAG-3'). PCR was performed with JumpStart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA). The conditions were as follows: 5 m at 94 ^{0}C and then 42 cycles of 30 s at 94 ^{0}C, 30s at 54 ^{0}C and 1 m at 72^{0}C followed by a final extension of 7 m at 72 ^{0}C. The PCR products were subcloned into the TOPO pCRII vector (TA Cloning Kit Dual Promoter, Invitrogen) and sequenced.

Whole mount in situ *hybridization*

A digoxigenin-labeled RNA probe was prepared by *in vitro* transcription of the zebrafish *iqgap2* cDNA fragment using the Roche DIG-RNA Labeling Kit (Roche Diagnostics, Rotkreuz, Switzerland). Zebrafish embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight and whole-mount *in situ* hybridization was performed as previously described [17] with following modifications: the embryos were treated with 10 µg/ml proteinase K for 20 min at room temperature; hybridization was performed overnight at 62°C; the anti-digoxigenin antibody (Roche Diagnostics) was diluted at 1:5000 with 1% Roche blocking reagent (Roche Diagnostics) in PBT (PBS with 0.1% Tween 20, Sigma-Aldrich).

Morpholino and RNA injections

Morpholino antisense oligonucleotides (GeneTools LLC, Philamath, Oregon, USA) were designed to target the translation start site (ATG-MO1 : 5'-GAGGCTTCACCATAAACTGCGCGCG-3' ; ATG-MO2 : 5'-GACTCTCGCTGCAAGATTCTTTCTC-3') and the exon 26 splice donor site (spMO : 5'-ATCCACCTTAGATCTGCCAGAGATG-3') of the zebrafish *iqgap2* mRNA transcript. The standard morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a control. The morpholinos were diluted in RNase free water with 0.1 % phenol red as an injection tracer and injected into fertilized embryos at 1-4 cell stages.

The pCMV-XL4 vector containing human IQGAP2 full-length clone was purchased from OriGene (Rockville, MD, USA). The IQGAP2 coding fragment was released from the vector together with T7 promoter by the restriction enzyme digestion with *Sma*I and *Sac*I (Roche Diagnostics). Capped and tailed RNA was *in vitro* transcribed using mMessage mMachine T7 kit (Life Technologies, Zug, Switzerland) and injected into embryos after phenol-chloroform purification.

Immunofluorescence

Rabbit polyclonal antibodies were generated against peptides corresponding to epitopes SWKGYKQRSVYKERLRT (aa795-811) and TNKFELLDGDDKDTKG (aa1379-1394) of zebrafish IQGAP2 and affinity purified at Eurogentec (S.A., Seraing, Belgium). A rabbit polyclonal antibody against zebrafish Nephrin was a kind gift from Dr. Arindam Majumdar (Uppsala University). Immunofluorescence was performed according to [18] at dilutions of 1:100 (α-iqgap2), 1:250 (α-Nephrin) and 1:500 for goat α-rabbit Alexa 568 (Invitrogen) as a secondary antibody. FITC-Phalloidin (Invitrogen) was used for co-staining of actin filaments at a dilution of 1:500.

Histology

Zebrafish larvae were fixed in 4% PFA overnight. Following a serial dehydration in ethanol, samples were infiltrated and embedded in Technovit 7100 (Kulzer Histotechnik, Wehrheim, Germany). Sections (3-5 µm) were cut on Leica RM 2145 and stained with Richardson solution (1% methylene blue, 1% Borax and 1% azur II, 1 : 1 : 2).

Glomerular filtration assay

Zeberafish larvae at 4dpf were anaesthetized with 200mg/ml Tricane. Lysin-fixable 70kDa TexasRed dextrans and 500kDa FITC-dextrans (Invitrogen) were dissolved in 150mM NaCl solution. 500kDa dextrans were filtered prior to injection by Amicon Ultra 15ml 100K (Millipore) to remove fragments

smaller than 100kDa. The dextran solution was injected into the blood circulation according to [19]. The larvae were fixed 6 hours post injection (hpi) and subsequently analyzed by Olympus BX61 wide-field microscope.

Electron microscopy

Zebrafish larvae at 72, 96 and 120 hpf were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 overnight (A tail of each fish was cut off in the fixative by a forceps to achieve better immersion of samples). The fish were then rinsed in 0.1 M cacodylate buffer prior to postfixation in 1 % Osmium tetroxide in cacodylate buffer for 30 min at room temperature. The samples were washed in cacodylate buffer followed by distilled water before en bloc staining in 1 % aqueous uranyl acetate for 30 min at room temperature. Following dehydration through a graded series of ethanol, the fish were infiltrated and embedded in Epon overnight at room temperature. Thin sections (65-70 nm) were cut on a Leica EM FCS ultramicrotome and collected onto formvar-coated copper grids. Grids were analyzed on a Philips CM 100 at 80 kV.

3.4. Results

Candidate gene selection

As the aim of the study was to identify gene transcripts potentially involved in the development of acquired forms of steroid-sensitive NS, we compiled from the literature a list of glucocorticoid receptor (GR)-regulated genes that were then matched with REGGED, a human renal glomerulus-enriched gene expression dataset [20], in order to identify GR-regulated and glomerulus-enriched genes. Of the 207 initially listed GR-regulated genes, 22 genes were glomerulus-enriched. To further narrow down the list of potential candidates, the regulation of the glomerular-enriched GR-associated genes in patients with NS was assessed using microarray data from micro-dissected glomeruli from patients with biopsy-proven NS (FSGS, MCD and MGN)[21]. Analysis of the gene expression profiles identified a number of genes dysregulated in steroid-sensitive NS. Only genes, which were found to be differentially regulated in a similar fashion in at least two separate disease datasets were selected. One dysregulated gene was the IQ motif containing GTPase activating protein 2 (IQGAP2). In the datasets, the expression of IQGAP2 was decreased to 76% in biopsies of patients with MCD and to 78% in patients with FSGS compared to control. Consistent with the mRNA data in the REGGED database, immunohistochemistry with an antibody against human IQGAP2 revealed that IQGAP2 is also highly abundant at the protein level in human glomerular podocytes (Fig. 1A). Given that IQGAP2 is one of the three evo-

lutionarily conserved IQGAP isoforms known to interact with and control the dynamics of the actin cytoskeleton [22] and given that the actin cytoskeleton is crucial for the normal podocyte function [3], IQGAP2 was selected for further analyses.

Expression of the zebrafish ortholgoue of IQGAP2 *in the zebrafish pronephros*

The zebrafish orthologue of IQGAP2 has the same protein domains as mammalian IQGAPs, including calmodulin-binding IQ motifs and a GTPase activating protein (GAP)-related domain (GRD) (Fig. 1B). The alignment of the putative amino acid sequence of zebrafish IQGAP2 with human IQGAP2 revealed that the zebrafish protein shares 57.9% sequence identity and 68.8% sequence similarity with the human counterpart (Supplementary Fig.1). Interestingly, the calponin homology domain (CHD) and the GRD display a very high degree of sequence homology (CHD; 92.2%, GRD; 85.2%), suggesting that there is a strong conservation pressure on these functional domains (Fig. 1B).

The whole mount *in situ* hybridization demonstrated a specific expression of *iqgap2* in the zebrafish pronephros. *iqgap2* transcripts were visible both in the glomerulus and tubules of the developing pronephros at 48hpf (Fig. 1C). However, iqgap2 expression levels were higher in the glomerulus than in the tubule (Fig. 1D). Using a custom made antibody raised against zebrafish IQGAP2, immunofluorescence showed a prominent abundance of Iqgap2 in podocytes of the pronephric glomerulus. Invading endothelial cells from the dorsal aorta or mesangial cells did not show any IQGAP2 immunoreactivity (Fig. 1E, F, G). Compared to the expression of Nephrin that is localized primarily along the GBM (Fig. 1H), Iqgap2 appears more widely distributed in podocytes. In line with the previous demonstration that IQGAP2 localizes to actin-rich structures [23], co-staining with fluorescein-labelled phalloidin showed some overlap of the Iqgap2 immunostaining with the actin staining by phalloidin (Fig. 1F, G).

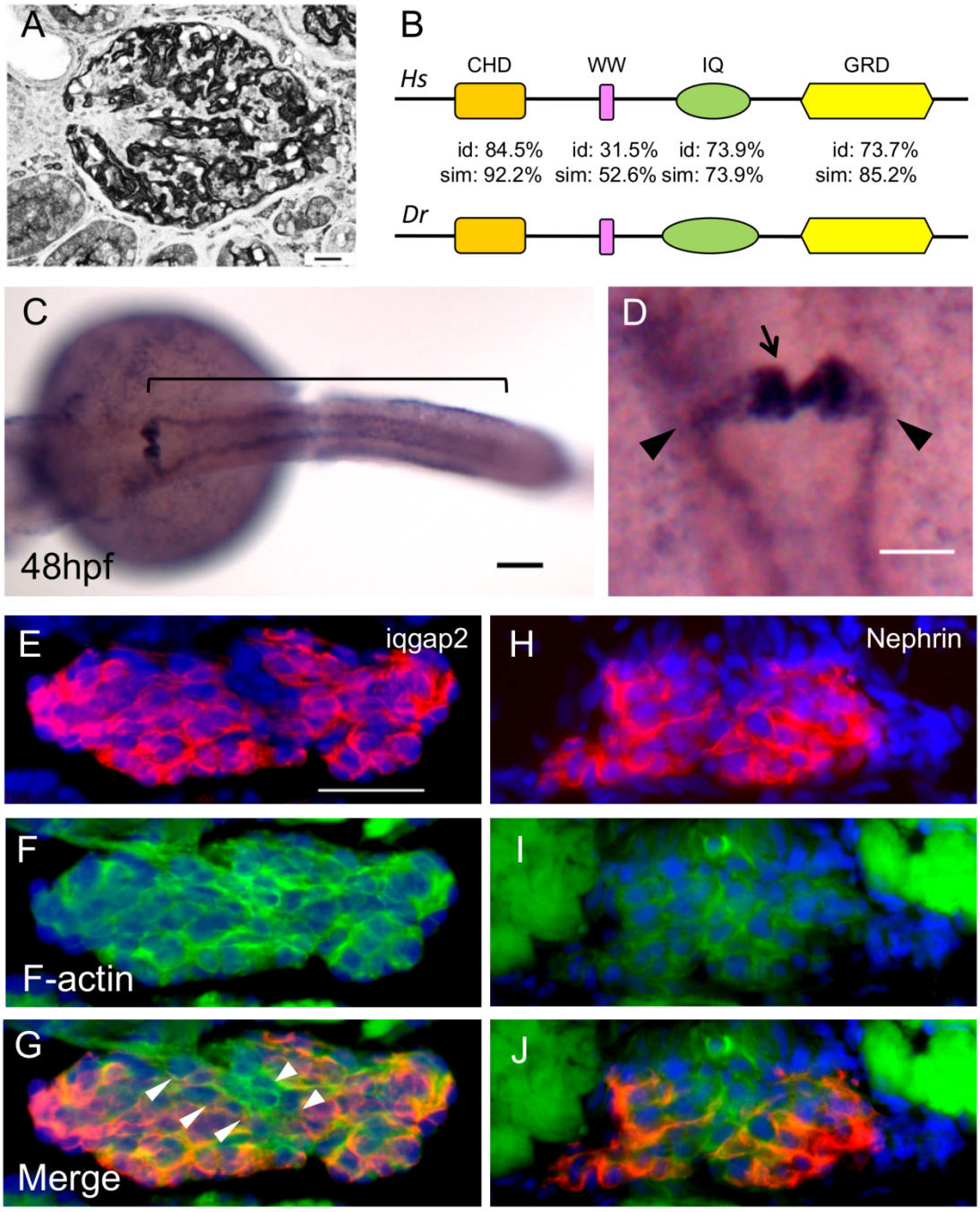

Fig. 1. *iqgap2* expression in the zebrafish pronephros. (A) Comparison of the predicted protein structure of zebrafish IQGAP2 with human IQGAP2. CHD, calponin homology domain; WW, poly-proline-binding domain; IQ, calmodulin-binding motif; GRD, Ras GTPase-activating protein (GAP)-related domain. Sequence identity (id) and similarity (sim) of each functional domain between human and zebrafish IQGAP2 are noted. (B) *iqgap2* mRNA expression is observed in the entire pronephros. Scale bar, 100 μm. (C) A higher magnification shows a glomerulus-enriched expression of *iqgap2* in the pronephros. The glomerulus (arrow); the tubules (arrowheads). Scale bar, 50 μm. (D)(E)(F) Immunofluorescence of Iqgap2 in the pronephric glomerulus with co-staining of F-actin by phalloidin. Iqgap2 is expressed in podocytes but is absent in endothelial cells and mesangial cells that show only the phalloidin staining (F; arrowheads). (G)(H)(I) Nephrin expression in podocytes. Scale bar, 30 μm.

iqgap2 *knockdown causes a glomerular expansion in zebrafish*

To obtain insights into the functional role of Iqgap2 in the zebrafish pronephros, we performed knockdown experiments of *iqgap2* by morpholino anti-sense oligonucleotides. Two morpholinos (ATG-MO1 and MO2) were designed to block translation of *iqgap2* as well as one splice morpholino (spMO) targeting the splice donor site of exon 26 to produce iqgap2 protein with a non-functional GRD (Fig. 2A). Immunofluorescence with the iqgap2 antibody on sections of *iqgap2* morphants did not show any iqgap2 protein expression in the glomerulus, confirming the efficient blocking of *iqgap2* translation by the ATG-MOs (Fig. 2B). Furthermore, RT-PCR demonstrated that the spMO indeed interfered with the splicing of the targeted exon, resulting in additional PCR products compared with the wild type mRNA transcripts (Fig. 2C). Sequencing of these extra PCR products suggested that they derived from either retention of the intron 25 or from skipping of exon 26 (Fig. 2D). In both cases, the altered mRNA transcripts are predicted to produce in-frame deletions of iqgap2 GRD.

The *iqgap2* morphants developed pericardial edema and exhibited an expanded glomerulus (Fig. 2E, F). Histological sections revealed that Bowman's space and the capillary loops were dilated in *iqgap2* morphants compared to the wild type larvae (Fig. 2G). To better visualize the glomerular morphology and to quantify the prevalence of the glomerular phenotype, the morpholino oligonucleotides were injected into *Tg(wt1b:*EGFP*)* embryos that express EGFP in the glomerulus and the surrounding proximal tubules [24]. The glomerular expansion was well evident in the *Tg(wt1b:*EGFP*)* larvae injected with the morpholinos (Fig. 2H). Prevalence of this phenotype in *Tg(wt1b:*EGFP*)* larvae was dose-dependent with both ATG-MOs (Fig. 2I). Co-injection of ATG-MO1 with human IQGAP2 mRNAs reduced the percentage of larvae with cystic dilation of the glomerulus. Thus, the glomerular phenotype of *iqgap2* morphants can be largely rescued by co-expression of intact human IQGAP2, suggesting that the *iqgap2* morphant phenotype is due to a specific effect of the used morpholinos (Fig. 2J).

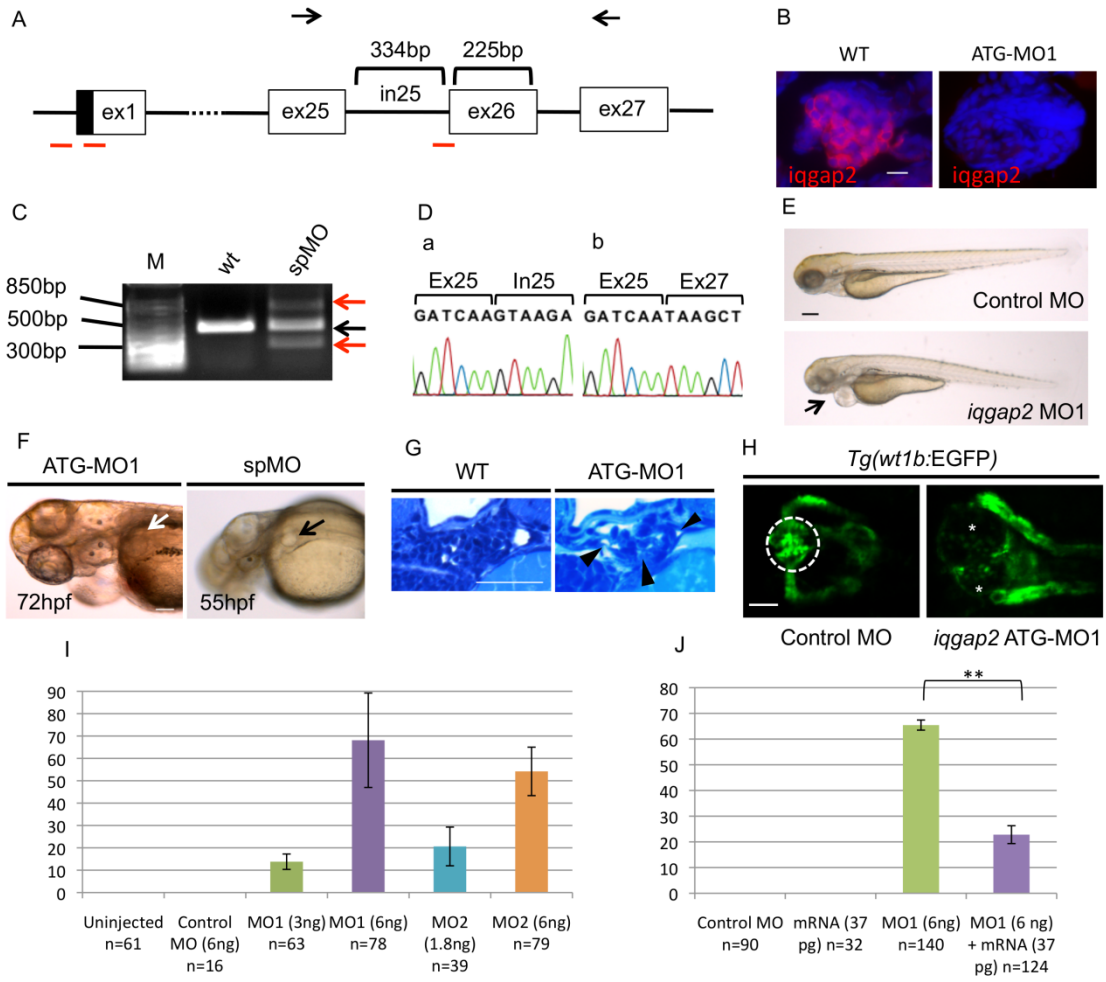

Fig. 2. Morpholino knockdown of *iqgap2* causes abnormal morphology of the glomerulus in zebrafish. (A) Exon structure of *iqgap2* around binding sites of *iqgap2* ATG-MO1, MO2 and spMO. Primers for RT-PCR are shown (arrow). The sizes of the intron 25 and exon 26 are indicated. (B) Absence of expression of iqgap2 in the glomerulus from iqgap2 ATG-MO1 injected larva at 4 dpf. Scale bars, 10 μm. (C) RT-PCR of *iqgap2* mRNA from iqgap2 spMO injected larvae yields extra bands (red arrows) in addition to a reduced band from iqgap2 wild type mRNA (black arrow). (D) Sequence of the extra PCR products reveal that the spMO causes retention of intron 25 (a) and skipping of exon 26 (b). (E) Gross morphology of control and *iqgap2* morphants at 2 dpf. Substantial fractions of *iqgap2* morphants develop pericardial edema (arrow); control MO: 14 %, ATG-MO1: 66 %, ATG-MO2: 81 %, spMO: 83 %. Scale bar, 200 μm. (F) *iqgap2* morphants exhibit an expanded glomerulus (arrows). Scale bar, 100 μm. (G) Histological sections of glomeruli from the wild type and *iqgap2* morphants. The Bowman´s space is larger in the morphant compared to the wild type and glomerular capillaries are dilated (arrowheads). Scale bar, 30 μm. (H) A glomerulus in the control and an expanded glomerulus in Tg(*wt1b*:EGFP) injected with *iqgap2* morpholinos. Scale bar, 30 μm. (I) A dose-dependent prevalence of the glomerular phenotype in Tg(*wt1b*:EGFP). (J) Decrease in the percentage of the glomerular phenotype in *iqgap2* morphants by co-injection of human IQGAP2 mRNAs. Three biological replicates were performed for statistical analysis. $^{**}P < 0.01$; Student's *t* test.

Normal development of the glomerulus in iqgap2 *morphants*

To test if the glomerular expansion in *iqgap2* morphants resulted from a developmental defect of the glomerulus, we investigated the development of the glomerulus in *iqgap2* morphants. An mRNA probe for Wilms tumor suppressor 1a (wt1a) was used to visualize the podocytes. Wt1 is an early acting transcription factor exclusively expressed in differentiating podocytes during nephrogenesis [25]. In zebrafish, glomerular primordia merge at the midline by 40 hpf before the urine filtration starts approximately at 48 hpf (Fig. 3A) [26]. In *iqgap2* morphants, expression of *wt1a* was similar to that of wild type embryos and the glomerular expansion started to appear only after the onset of the glomerular filtration (Fig. 3B). This observation suggests that knockdown of *iqgap2* neither affects the differentiation of podocytes nor the gross development of the glomerulus. Equally visible presence of *podocin* and *podocalyxin* positive cells both in control and morphant larvae further supports this conclusion (Fig. 3C).

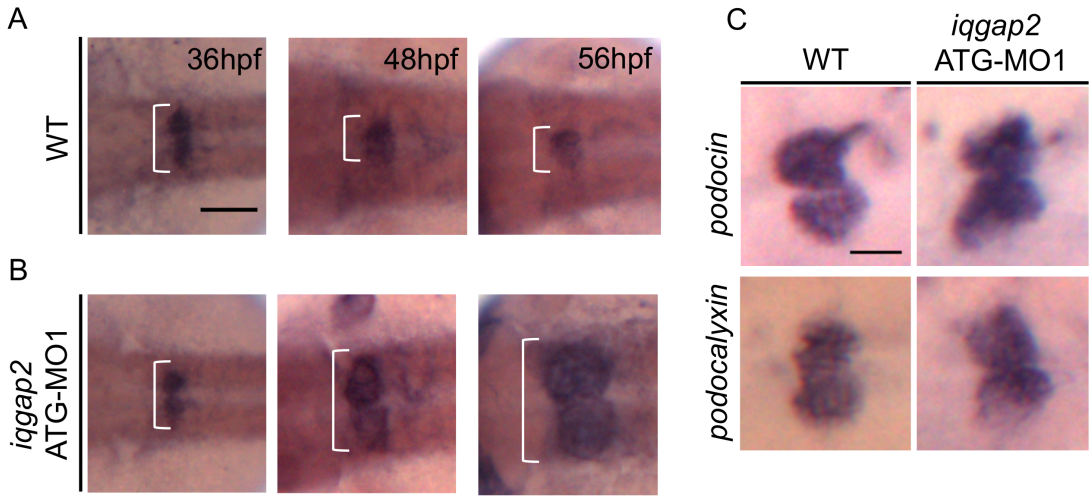

Fig. 3. Normal development of the pronephric glomerulus in *iqgap2* morphants. (A) A glomerular development in wild types visualized using *wt1a*, podocyte differentiation marker. The glomerular primordia (brackets) merge at the midline before the filtration takes place at 48 hpf. (B) In *iqgap2* morphants, the glomerular apparatus merge normally at the midline. After the onset of filtration, the glomerulus starts to expand (brackets). (C) Expression of *podocin* and *podocalyxin* in wild types and *iqgap2* morphants. Scale bars, 100 μm.

Deficiency of iqgap2 results in a leaky glomerulus

Next, we analyzed glomerular function in *iqgap2* morphants. To assay the size- selectivity of the glomerular filter, 70kDa TexasRed and 500kDa FITC dextrans were coinjected into the circulation of

wild types and *iqgap2* morphants. The size selectivity of the GFB is around 70kDa, which approximately equals the size of serum albumin. Both in the wild type and *iqgap2* morphants, fluorescent signals from the injected 70kDa dextrans were observed in the apical endosomes of the proximal tubules. The fluorescence signal appeared to be more pronounced in the morphants than in controls (Fig. 4A, B, C). In contrast to the 70kDa dextrans, co-injected FITC-500kDa dextrans were seen only in the proximal tubules of *iqgap2* morphants but not in the wild type larvae (Fig. 4A´, B´, C´). This difference suggests that the function of the glomerulus in *iqgap2* morphants is impaired, allowing the passage of macromolecules with a size exceeding the size selectivity of an intact glomerular filtration barrier.

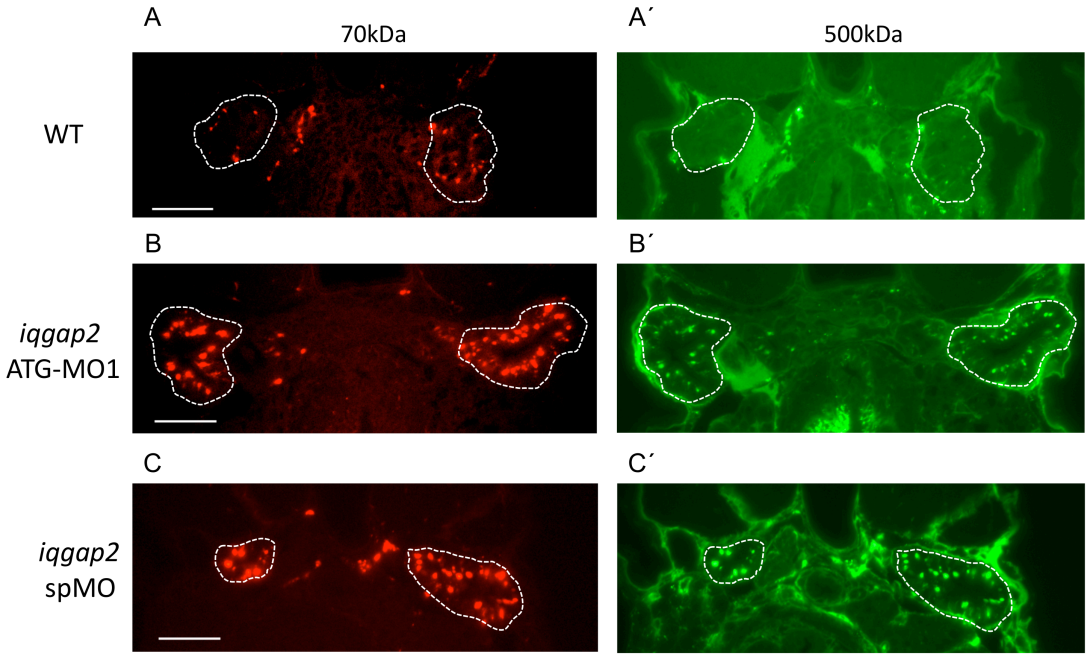

Fig. 4. Impaired glomerular filtration in *iqgap2* morphants. (A)(A´) A cross section of a 4dpf wild type larva at the level of the proximal tubule shows an uptake of TexasRed 70kDa dextrans while an absence of 500kDa FITC dextrans. (B)(B´)(C)(C´) Sections of 4dpf larvae injected with *iqgap2* ATG-MO1 and spMO exhibit a presence of 500kDa dextrans in the tubular passage. Scale bars, 30μm.

Normal development and function of the pronephric tubule in iqgap2 *morphants*

As *iqgap2* is also expressed in the pronephric tubule, we examined the development and function of the pronephric tubule in *iqgap2* morphants. First, developmental expression of *pax2a* was investigated. *pax2a* is expressed in the developing renal tubule from an early stage, acting as a determinant of the tubular fate of the developing kidney [27]. In *iqgap2* morphants, *pax2a* was expressed as in wild type larvae, suggesting that the tubular development is not impaired in the mophants (Fig. 5A). The

tubular morphology in *iqgap2* morphants was also examined by expression of *cdh17.* No recognizable difference in expression of *cdh17* was seen between wild type and morphant larvae (Fig. 5B). Furthermore, the segmentation of the pronephric tubule was investigated using segment specific markers for the proximal convoluted tubule (PCT; *slc20a1a*), proximal straight tubule (PST; *trpm7*), distal early (DE; *slc12a1*), distal late (DL; *clcnk*), corpuscle of Stannius (CS; *gata3*), collecting duct (CD; *gata3*) and the cloaca (C; *aqp3*) [28]. Again, the expression patterns of these markers were similar between *iqgap2* morphants and wild type larvae (Fig. 5C). We also explored the morphology of epithelial cells of the proximal tubule in *iqgap2* morphants at the ultrastructural level. Epithelial cells of the proximal tubule of *iqgap2* morphants exhibited a polarized morphology with well-developed brush borders on the apical surface (Fig. 5D). Moreover, the endocytic apparatus, including endosomes and dense apical tubules, was also present in the apical aspect of the epithelial cells, indicative of the normal function of the proximal tubule in *iqgap2* morphants, which was also evidenced by the active uptake of the filtered dextrans (Fig. 4). Although normal in tubular development and function, *iqgap2* morphants exhibited a slight tubular dilation (Fig. 5E). This tubular dilation may be due to the impaired function of the glomerular filtration barrier, creating an excess fluid delivery to the glomerular space and tubular lumen. Alternatively, the dilation may be caused by debris (e.g. dying cells, protein casts) from the damaged glomerulus, as seen in *nephrin* and *podocin* morphants [29]. In fact, some debris was occasionally detected in some renal tubules of morphants (Supplementary Fig. 2A). A cystic dilation of renal tubules has also been reported to be caused by a malformation of the primary cilium on the tubular epithelial cells [30]. However, immunohistochemical detection of acetylated tubulin showed a normal formation of primary cilia in both wild types and *iqgap2* morphants (Supplementary Fig. 2B).

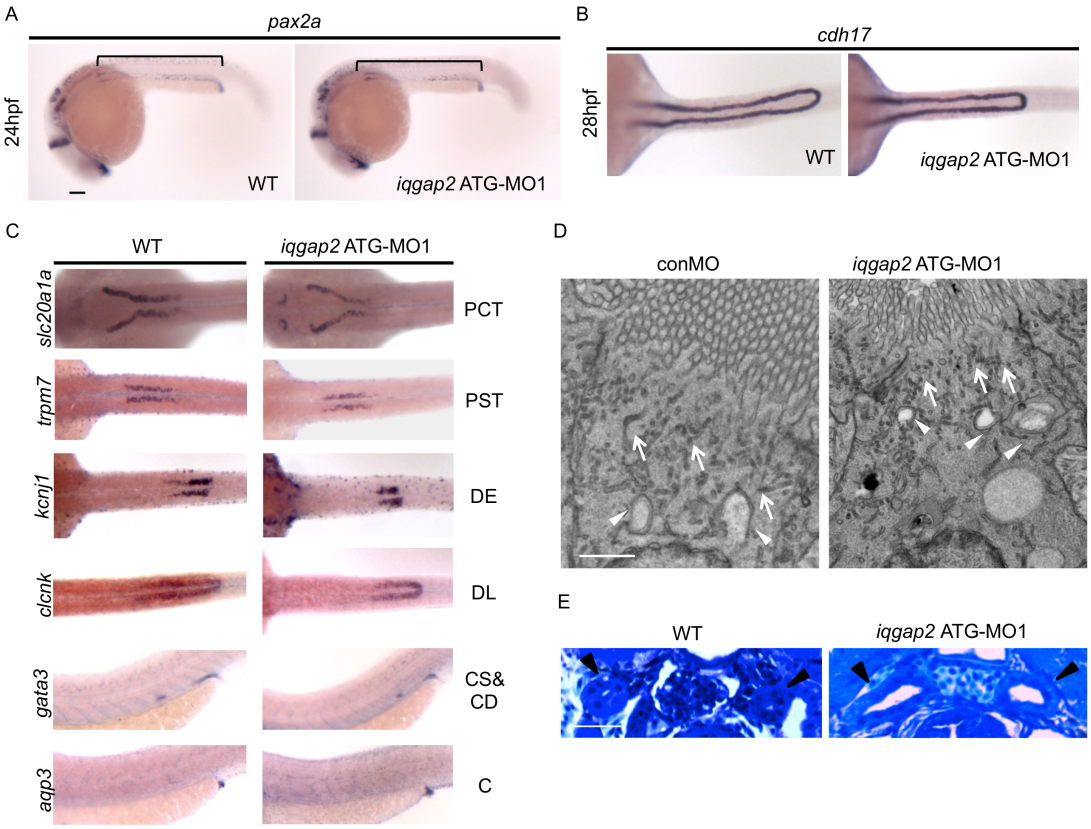

Fig. 5. Normal development and morphology of the pronephric tubule in *iqgap2* morphants. (A) Developmental expression of *pax2a* does not differ between wild types and *iqgap2* morphants. Scale bar, 100 μm. (B) mRNA expression of *cdh17* in *iqgap2* morphants is comparable to that of wild types (C) Segmentation of the pronephric tubule in *iqgap2* morphants. No detectable changes in the markers for all the tubular segments in *iqgap2* morphants. PCT; proximal convoluted tubule, PST; proximal straight tubule, DE; distal early, DL; distal late, CS; corpsucle of Stannius, CD; collecting duct, C; cloaca. (D) The ultrastructure of epithelial cells of the proximal tubule in the control and *iqgap2* morphants at 4dpf. The polarized feature is evident in *iqgap2* morphants with the apical brush border. Endocytic apparatus, such as endosomes (arrowheads) and dense apical tubules (arrows) are also present in the morphants. Scale bar, 1 μm. (E) Tubular dilation in *iqgap2* morphants (arrowheads). Scale bar, 30 μm.

Progressive effacement of podocyte FPs in iqgap2 *morphants*

The characteristic pathological feature of NS is effacement of podocyte FPs at the ultrastructural level [1]. To determine if the failure of the glomerular filtration in *iqgap2* morphants is associated with effacement of podocyte FPs, we next conducted electron microscopic analysis of the structure of the GFB. Although the glomerular filtration starts at 48 hpf in zebrafish, the size selectivity of the GFB is not yet fully developed until around 96 hpf. Accordingly, podocyte FPs were still immature and SDs

were hardly visible at 72 hpf both in wild type and *iqgap2* morphants (Fig. 6A, B). From 96 hpf on, the GFB acquires the mature organization and shows the typical thin podocyte FPs interconnected by SDs on the outer side and with fenestrated ECs on the inner side of the GBM (Fig. 6C, E). At 96 hpf, the GFB in *iqgap2* morphants exhibited a heterogeneous morphology with regions that appeared similar to the GFB in control larvae, but also with areas that showed effaced podocyte FPs (Fig. 6D). These phenotypic features progressed towards 120 hpf. At this point, effaced podocyte FPs were the dominant feature and intact SDs were only rarely observed in *iqgap2* morphants (Fig. 6E). Moreover, the fenestration of ECs was reduced in *iqgap2* morphants compared to wild types.

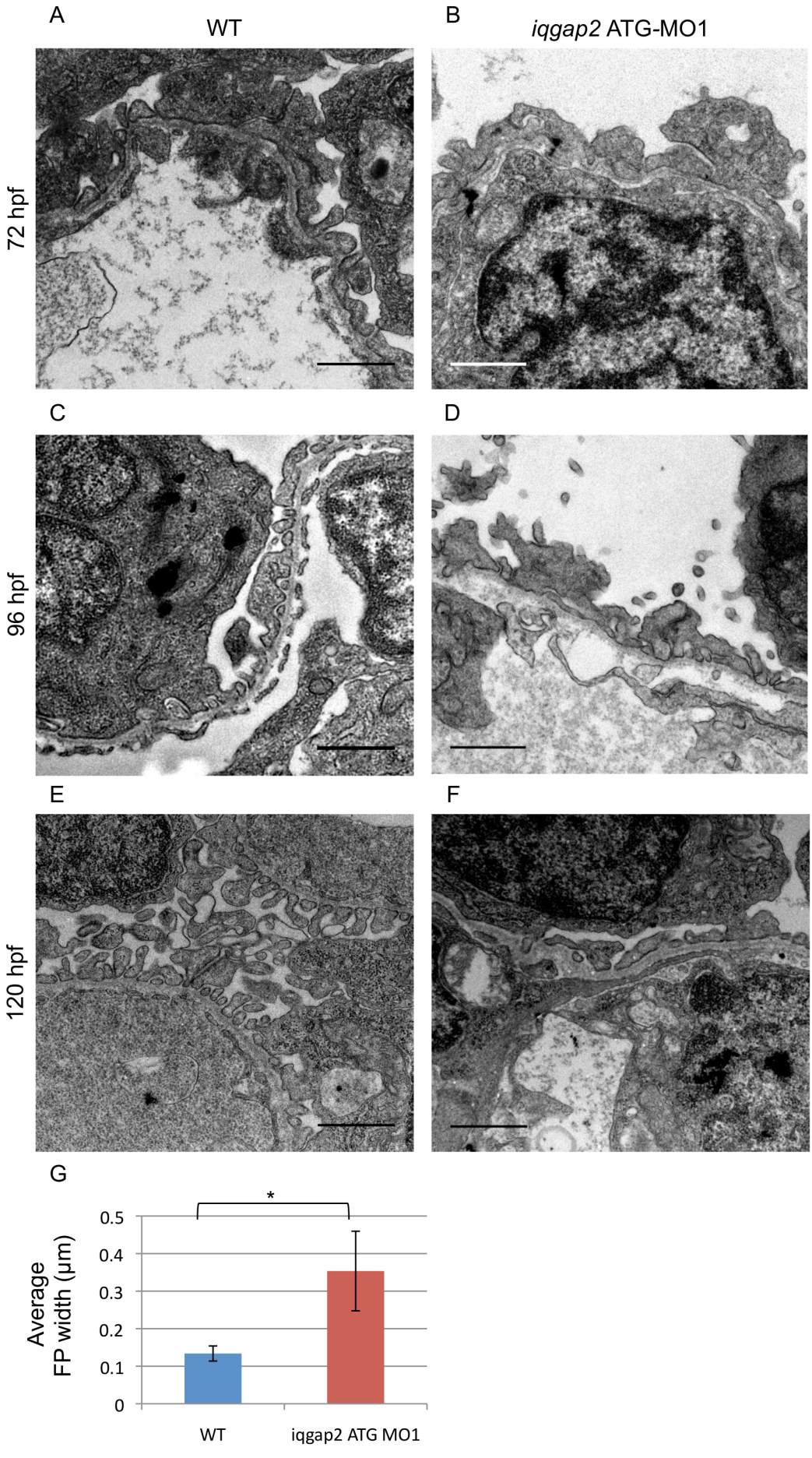
A
WT
B
iqgap2 ATG-MO1
72 hpf
C
D
96 hpf
E
F
120 hpf
G
Average FP width (µm)
0.5
0.4
0.3
0.2
0.1
0
*
WT
iqgap2 ATG MO1

Fig. 6. Ultrastructural changes of the GFB in *iqgap2* morphants. (A)(B) Electron microscopy of the GFB at 72 hpf. Both in wild types and *iqgap2* morphants, podocyte FPs are still immature at this stage. (C)(D) Mature podocyte FPs are present along the GBM with fenestrated ECs at 96 hpf, but in the morphants, areas with thin podocyte FPs are reduced compare to wild types. (E)(F) At 120 hpf, in contrast to fully developed podocyte FPs in wild types, a greater fraction of podocyte FPs is effaced in *iqgap2* morphants. (G) Quantification of width of podocyte FPs in wild types and *iqgap2* motphants at 120 hpf. $*P < 0.05$; Student's *t* test. Scale bars, 1 μm.

3.5. Discussion

A comprehensive transcriptional analysis of renal biopsies from patients with NS can be a useful resource for the assessment of the complex regulatory network of podocytes [31]. In this study, we used such transcription data to identify novel potential candidate genes involved in the pathogenesis of NS and tested for their functional relevance by using the zebrafish as an easy-to-use assay system. Due to the short generation time and the ease of genetic manipulations, the zebrafish circumvents limitations of mouse models for rapid functional analyses of genes of interest [32]. This approach successfully identified IQGAP2 as a new regulator of podocyte structure and function.

IQGAPs are known as Rho GTPase binding proteins that associate with actin filaments and regulate numerous cellular processes involving the actin cytoskeleton, such as cell-cell adhesions and cell migrations [23][33]. We found that IQGAP2 is highly abundant in human podocytes and disruption of the zebrafish orthologue of IQGAP2 leads to impaired glomerular filtration barrier associated with a progressive effacement of podocyte FPs, similar to what is seen in human patients with NS. Although the zebrafish *iqgap2* starts to be expressed from an early stage of development, knockdowns of *iqgap2* do not affect development of the glomerulus. Podocytes normally differentiate in *iqgap2* morphants and an abnormal morphology of the glomerulus starts to form after the onset of the filtration only. This is distinct from morphants for *wt1a*, a key factor in the developing kidney, where podocytes fail to differentiate and expressions of *nephrin* and *podocin* are absent [24]. Moreover, although, in *iqgap2* morphants, a substantial fraction of FPs is effaced, the epithelial cell polarity with an apical and basolateral domain is well established in podocytes that are attached to the seemingly normal GBM, suggesting that the formation of the GFB normally takes place. This is further supported by the observation that SDs are still present between the remaining thin FPs in *iqgap2* morphants. Therefore, we conclude that iqgap2 does not play a significant role in glomerular development and maturation but in the maintenance of a highly differentiated podocyte structure important for the normal function of the GFB.

We also analyzed the recently published *Iqgap2* total knockout mice [34]. In contrast to the zebrafish morphants, these mice did not show any nephrotic phenotype. However, this does not exclude that

Iqgap2 participates in the podocyte regulation. Functional redundancies with other Iqgap isoforms may compensate for the loss of the Iqgap2 function in the kidney from the *Iqgap2 -/-* mice. Likewise, a constitutive knockout of a gene during development might be better compensated than an acute knockdown (e.g. by morpholinos). Moreover, an incomplete loss-of-function, as achieved with morpholino knockdowns, may fail to activate a sufficient upregulation of compensatory pathways. For instance, mice with a heterozygous disruption of the insulin responsive glucose transporter, *Glut4*, reveal a severe diabetic phenotype whereas mice with a homozygous disruption of *Glut4* are only mildly affected, likely due to compensation for the loss of Glut4 by other glucose transporting mechanisms [35]. Therefore, it would be an important future step to study inducible *Iqgap2* knockout mice for further characterization of IQGAP2 in the pathogenesis of NS.

The highly dynamic organization of podocytes is supported by the elaborate architecture of the actin cytoskeleton. The Rho GTPases, Rac1, Cdc42 and RhoA, regulate this dynamic structure of podocytes by controlling the actin cytoskeleton [36]. A podocyte specific loss of Cdc42 causes congenital nephropathy in mice whereas a transgenic mouse with constitutively active Rac1 develops proteinuria with effacement of podocyte FPs [37][38]. Given its role as an effector of the Rho GTPases, IQGAP2 may be one of the mediators of the Rho GTPases in podocytes, thereby contributing to the maintenance of the filtration function of the GFB. Notably, also the closely related protein IQGAP1 was reported to interact with the podocyte cytoskeleton and components of the SD, including Nephrin [39], and to control podocyte motility and permeability *in vitro* [40]. Together with a recent report on downregulation of IQGAP1 in patients with diabetic nephropathy [41], both IQGAP isoforms may play a particular role in podocyte biology. Future experiments will have to determine whether IQGAP1 and IQGAP2 possess redundant and/or unique functions and how they are possibly linked to acquired and/or hereditary forms of NS in humans. Genetic polymorphisms have been reported for IQGAP2, but they were associated with hearing impairments and altered triglyceride levels under thiazide treatment [42][43].

In summary, we identified IQGAP2 as a human and zebrafish podocyte gene that is downregulated in microarray datasets from kidney biopsies of human patients with FSGS and minimal change NS. By using the zebrafish glomerulus as an easy-to-manipulate assay system, we provide morphological and functional evidence that IQGAP2 is critical for normal podocyte structure and function, hence maintaining the GFB integrity.

Acknowledgements

The authors would like to thank following researchers; Dr. Christoph Englert (Leibniz Institute for Age Research, Jena, Germany) for the Tg(*wt1b*:EGFP) line, Dr. Arindam Majumdar (Uppsala University) for the zebrafish Nephrin antibody and Dr. Valentina Schmidt (Stony Brook University Medical Center) for the *Iqgap2* -/- mice. The authors also wish to thank Kerstin Dannenhauer for her excellent care of the zebrafish lines, Dr. Jose Maria Mateos and Therese Bruggmann (Center for Microscopy and Image Analysis, University of Zurich) for their technical support for electron microscopy and Ilka Edenhofer for immunohistochemistry on human biopsies.

We thank all participating centers of the ERCB-KFB; the centers are listed at; http://www.research-projects.uzh.ch/p9291.htm. The ERCB-KFB is supported by the Else Kröner-Fresenius Foundation.

The study was supported by a cooperative project grant from the Zurich Center for Integrative Human Physiology at the University of Zurich and EURenOmics, European Consortium for High-Throughput Research in Rare Kidney Diseases (EU FP 7: 305608). Additional support came from project grants from the Swiss National Science Foundation (SN, JL) and the National Center of Competence in Research Kidney.CH (JL). SS is supported by the "Fundação Pesquisa e Desenvolvimento Humanitário"

Supplementary information is available online at http://www.nature.com/ki

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Chapter 4

Functional studies of thiazide-sensitive NaCl cotransporter in the zebrafish pronephros

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Report on an ongoing research

Personal contributions:

Antibody development, whole-mount immunostaining, western blot analysis, *in situ* hybridization, morpholino knockdowns except for ppp1r1b, salinity treatment, preparation of all figures except for Fig. 3 and writing of the manuscript.

4.1. Abstract

The distal convoluted tubule (DCT) that expresses thiazide-sensitive NaCl cotransporter (NCC) plays a critical role in ion homeostasis in mammalian kidneys. However, the hideous localization of the DCT makes this nephron portion inaccessible for direct functional analyses. In addition, there are no cell lines that allow *in vitro* studies of NCC in the proper cellular context. Thus, a new model system in which NCC can be investigated *in vivo* is desired. The zebrafish model provides an easily accessible nephron with a high degree of genetic conservation with the mammalian nephron. In this study, we developed antibodies that detect total NCC abundance and phosphorylation status of NCC. The zebrafish NCC (zNCC) proteins localize to the distal late (DL) segment of the pronephros, the corresponding segment of the mammalian DCT. The phospho-forms of zNCC reside on the apical surface of the DL cells, suggesting its conserved function in reabsorption of NaCl. More importantly, abundance of phospho-zNCC changes in response to a hypertonic stress as well as upon knockdowns of its putative regulator, presenting a proof-of-principle that the zebrafish pronephros can serve as a model system to study NCC activity *in vivo*.

4.2. Introduction

The mammalian distal nephron plays a critical role in the regulation of NaCl homeostasis and the control of blood pressure [1]. This essential function of the distal nephron is achieved by transporters in the apical membranes of epithelial cells along this nephron segment. The furosemide-sensitive NaKCl2 cotransporter (NKCC2) and the thiazide-sensitive NaCl cotransporter (NCC) are the major pathways for the apical sodium transport in the thick ascending limb (TAL) and the distal convoluted tubule (DCT), respectively. Genetic impairment of these transporters leads to hereditary diseases with pronounced alterations in blood pressure. In fact, although a substantial fraction of sodium is reabsorbed by the proximal nephron, almost all causative genes for monogenic disorders with dysregulated ion homeostases converge on genes encoding for transporters in the distal nephron [2]. Loss-of-function mutations of NCC cause Gitelman syndrome (GS) characterized by hypotension whereas hyperactivity of NCC results in familial hyperkalemic hypertension (FHHt), underlying the particular importance of this transporter in regulation of NaCl handling [3].

Characterization of GS and FHHt using rodent models and *in vitro* heterologous systems advanced our knowledge on NCC regulation. In particular, research using genetically modified rodent models has provided insights into the complex regulation of NCC. However, a better understanding of detailed mechanisms of the regulation of NCC has been hindered by lack of appropriate systems to study NCC *in vivo* [4]. The hideous location of this short nephron portion deep within the kidney cortex makes this segment inaccessible for direct functional analysis and live imaging. Furthermore, there are no cell lines available that allow for studies of NCC regulation in the proper cellular context. Therefore, a new model system that permits *in vivo* analyses of NCC is desired.

The zebrafish pronephros has been increasingly used as a genetic model system to study human kidney disease [5]. The zebrafish model system offers an array of useful genetic tools, such as morpholino antisense oligonucleotide for gene knockdowns and, TALEN and CRISPR/Cas9 system for gene knockout approaches [6][7]. Moreover, the high fecundity and short generation span make this animal model suitable for high-throughput assessments of gene function [8]. Also, owing to the ease of transgenesis, together with transparency of its embryos, the zebrafish is an excellent model for live imaging to monitor biological phenomena *in vivo* [9].

The zebrafish pronephric tubule consists of distinct segments likely with different functions in modifications of the urinary waste [10]. As in the metanephros, the pronephric proximal tubule recovers a bulk of macromolecules [11]. Function of the distal tubule of the pronephros is less clear but several lines of evidence suggest that the distal segments participate in fine-tuning of ion contents of the filtrate as do the mammalian counterparts [12][13]. Furthermore, the zebrafish possess the orthologues of most of the transporters identified as causative for diseases with dysregulated ion balance in humans, including NCC, and significantly, those orthologues show almost the same segmental localization in the pronephric tubules. This observation strongly indicates that, despite the difference in habitas, mechanisms of renal regulation of ion homeostasis are fundamentally conserved.

In the present study, to the aim of establishing the zebrafish pronephros as a system to analyse NCC activity *in vivo*, we developed antibodies against the total as well as two of the conserved phosphorylation sites of zebrafish NCC (zNCC). zNCC proteins specifically localize to the distal late (DL) segment of the pronephros that corresponds to the mammalian DCT. More importantly, using the phospho-zNCC anotibodies, we could detect changes in phosphorylation status of zNCC in the zebrafish pronephros treated with different salinities as well as in the zebrafish morphants for a putative NCC regulator.

4.3. Materials and methods

Zebrafish

Zebrafish were maintained under a 14h/10h light/dark cycle. Fish were bred as previously described and the embryos were raised at 28 ^{0}C in E3 medium [14].

Zebrafish NCC antibodies

Antibodies were raised in rabbit against aa290-307 (TPQKQARGFFSYRADIF) for the total zNCC, Thr 49 and Thr 62 for the phospho-zNCC. The antibodies were subsequently affinity purified at Pineda Ab-production (Berlin, Germany).

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was carried out as described elsewhere (Chapter 3).

Western blot analysis

Zebrafish lysates for Western blot analysis were prepared from adult zebrafish kidney. Four adult zebrafish kidneys were homogenized by sonication in an ice-cold K-HEPES buffer (200 mM mannitol, 80 mM K-HEPES, 41 mM KOH, pH 7.5) with a protease inhibitor and phosphatase inhibitor (Roche Diagnostics, Rotkreuz, Switzerland) [15]. The samples were centrifuged at 4.6 rpm for 15min at 4 ^{0}C. The loading buffer was added to the samples, which were then vortexed before Western blot analysis. SDS-PAGE was performed on 10 % polyacrylamide gels and subsequently proteins were blotted to Invitrolon PDVF membranes (Invitrogen, Basel, Switzerland). After blocking in 0.05 % PBST (1 X PBS, 0.05 % Tween 20) with 3 % milk powder for 1.5 hours, the blot were incubated with primary antibodies overnight at 4 ^{0}C with following dilutions; 1:1000 and 1:2000 (pT62-zNCC), and 1:3000 (β-actin). The blots were washed and blocked, and then incubated with the HRP-conjugated secondary antibodies (1:10000, anti-rabbit and anti-mouse, Jackson ImmunoResearch) for 2 hours at room temperature. Antibody binding was detected using ECL solution (Thermo Scientific). The blots were exposed for 45 min in ImageQuant LAS-4000 imaging system (GE Healthcare Lifescience). The images were analyzed and adjusted using ImageJ (NIH, Bethesda, USA).

Whole-mount immunofluorescence

Zebrafish embryos and larvae were fixed in 4% PFA for 30 min at room temperature (RT). Fixed larvae were serially dehydrated and stored in 100% MeOH at -20 ^{0}C at least overnight. Larvae were treated with acetone for 7 min at -20 ^{0}C and after washes in 1 X PBST (1 X PBS, 0.1 % Tween 20), blocked in the blocking buffer (1% BSA, 1% DMSO, 0.1% Tween 20, 5% normal goat serum in 1 X PBS) for 4 hours at RT. Larvae were then incubated with the respective primary antibodies in the blocking buffer at 4 ^{0}C overnight. Dilutions of the primary antibodies were as follows; 1:500 (t-zNCC, pT49-zNCC, pT62-zNCC) and 1:100 (α-Na-K-ATPase). Next day, larvae were washed in 1 X PBST several times and subsequently blocked in the blocking buffer for 3 hours at RT. Secondary antibodies were applied in 1 X PBST with a dilu-

tion of 1:500 at 4 ^{0}C overnight. Larvae were washed in 1 X PBST and 1 X PBS and stored in glycerol at 4 ^{0}C.

Immunohistochemistry

Immunofluorescence on sections of zebrafish larvae and kidneys from adult zebrafish was performed according to [16]. Antibodies were diluted at 1:500 (tzNCC, pT49-zNCC, pT62-zNCC), 1:100 (α-Na-K-ATPase) and 1:500 for goat α-rabbit Alexa 568 and goat α-mouse Alexa 488 (Invitrogen, Basel, Switzerland) as a secondary antibody.

Zebrafish salinity treatment

The zebrafish larvae were transferred from freshwater E3 medium to 50% seawater. 100 g KCl was dissolved in distilled water to make 130 mM KCl solution.

Morpholino injection

Morpholino antisense oligonucleotides (GeneTools LLC, Philamath, Oregon, USA) were designed to target the translation start sites (*slc12a1*-ATG-MO : 5'-AGGCTTTATTTGTTTGACTTGTAAA -3' ; *ppp1r1b*-ATG-MO : 5'-GGATCCATAATGCGCTTTCGTCCTC-3') of the zebrafish *slc12a1* and *ppp1r1b*. The standard morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a control. The morpholinos were diluted in RNase free water with 0.1% phenol red as an injection tracer and injected into fertilized embryos at 1-4 cell stages.

4.4. Results

Characterization of the zNCC antibodies

To obtain a tool to detect total zNCC, an antibody was raised against an extracellular domain of zNCC (tzNCC). Furthermore, in order to analyze NCC phosphorylation and hence NCC activity, antibodies were raised against two of the conserved phosphorylation sites [Thr 49 (pT49-zNCC) and 62 (pT62-zNCC) corresponding to Ser 60 and Thr 73 of human NCC, respectively]. Whole-mount immunofluorescence was performed to test the immunoreactivity of these an-

tibodies. The staining pattern of the developed antibodies clearly overlapped with the *in situ* hybridization staining of zNCC mRNAs that is restricted to the DL (Fig. 1A).

Western blotting with pT62-zNCC antibody revealed a single weak band of about 200 kDa in molecular mass in proteins extracted from adult zebrafish kidneys (Fig. 1B). tzNCC and pT49-zNCC did not yield satisfactory outcomes from Western blotting analysis. In order to determine the subcellular localization of zNCC in the DL cells, we conducted immunohistochemistry on sections of both the larval pronephros and the adult mesonephros. This analysis demonstrated the apical localization of phospho-zNCC, which is consistent with the localization of mammalian NCC in the DCT (Fig. 1C).

Interestingly, the immunostaining by pT49-zNCC extends towards the distal early (DE) segment that corresponds to the mammalian TAL (Fig. 1D). Considering the sequence similarity around the phosphorylation site, this ectopic staining outside the DL is likely a cross-reaction of this phospho-antibody with phospho-NKCC2 in the DE. This cross-reaction was confirmed by morpholino knockdowns of *slc12a1*, encoding for NKCC2, which abolished the staining in the DE while left the staining in the DL unchanged.

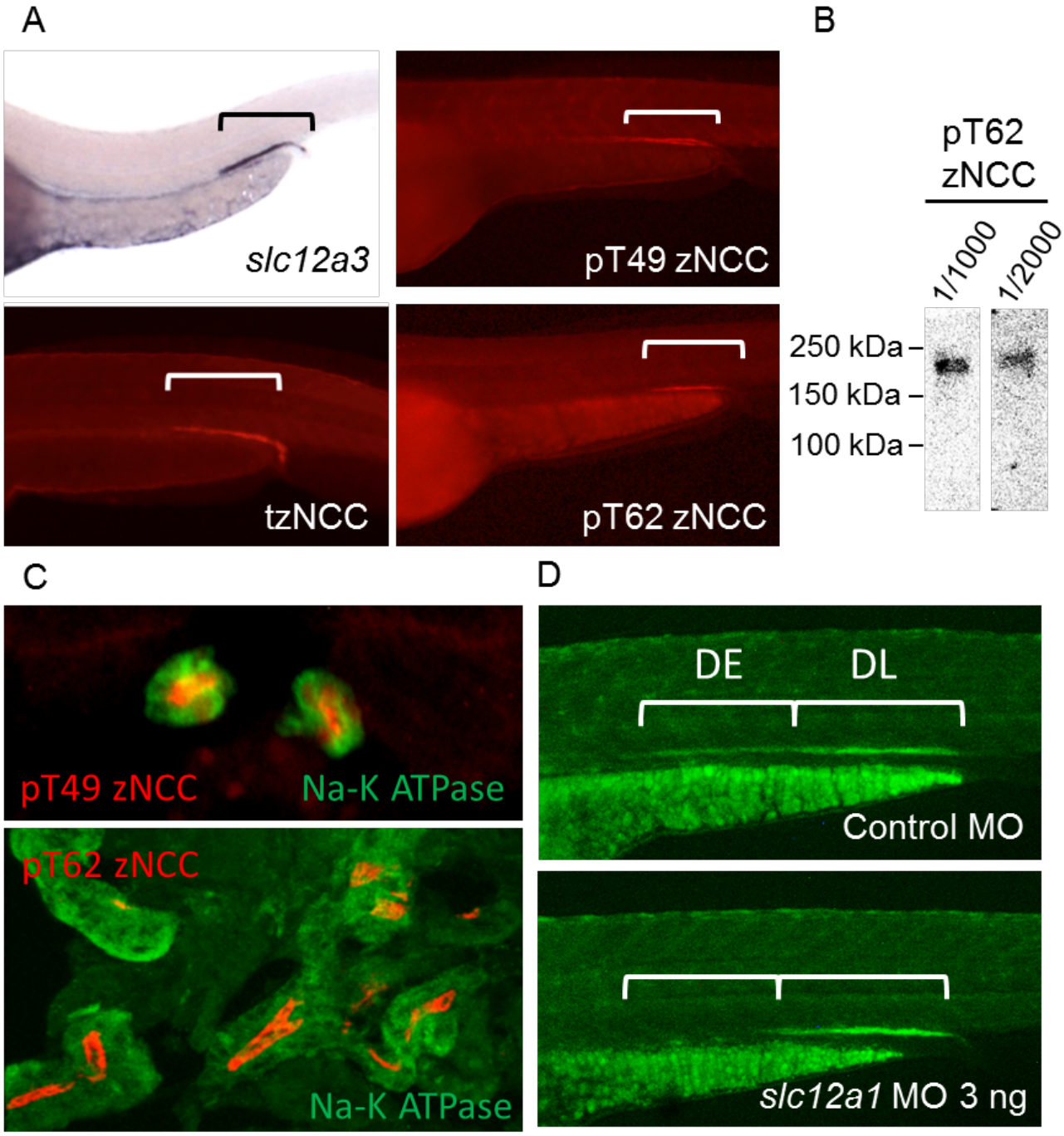


Fig. 1. Antibodies against total and phosphorylated forms of zNCC. (A)Total and phospho-zNCC in the pronephros detected by tzNCC, pT49-zNCC and pT62-zNCC. (B) In adult kidney homogenates, pT62-zNCC antibody detects a weak single band of the size of 200 kDa (arrow). (C) Apical localization of phosphorylated zNCC with basolateral staining of Na-K-ATPase in the pronephros and mesonephros. (D) Cross-reaction of pT49-zNCC with NKCC2. Morpholino knockdowns of *slc12a1* encoding for NKCC2 abolishes staining of pT49-zNCC in the DE while leaves the staining in the DL intact.

Decrease in phosphorylation status of zNCC upon hypertonic stress

Next, we sought whether the known modulators of the NCC activity in mammals could also change the activity of zNCC. We first explored effects of different environmental salinities in order to determine if the phospho-antibodies developed in this study can detect changes in phosphorylation status of zNCC. To this aim, we exposed zebrafish larvae to a hypertonic stress and subsequently performed immunofluorescence to analyze zNCC phosphorylation. The abundance of pT62-zNCC decreased in larvae treated with 20 % seawater overnight compared to untreated control larvae (Fig. 2). Furthermore, in larvae treated with 50 % seawater, fluorescent signals from this antibody almost disappeared. Since a recent study demonstrated that high K load rapidly dephosphorylate NCC in the DCT in the mouse kidney, we also tested effects of high concentration of KCl in the medium on phosphorylations of zNCC [17]. However, high KCl treatment did not cause a major change in the staining of the phospho-zNCC.

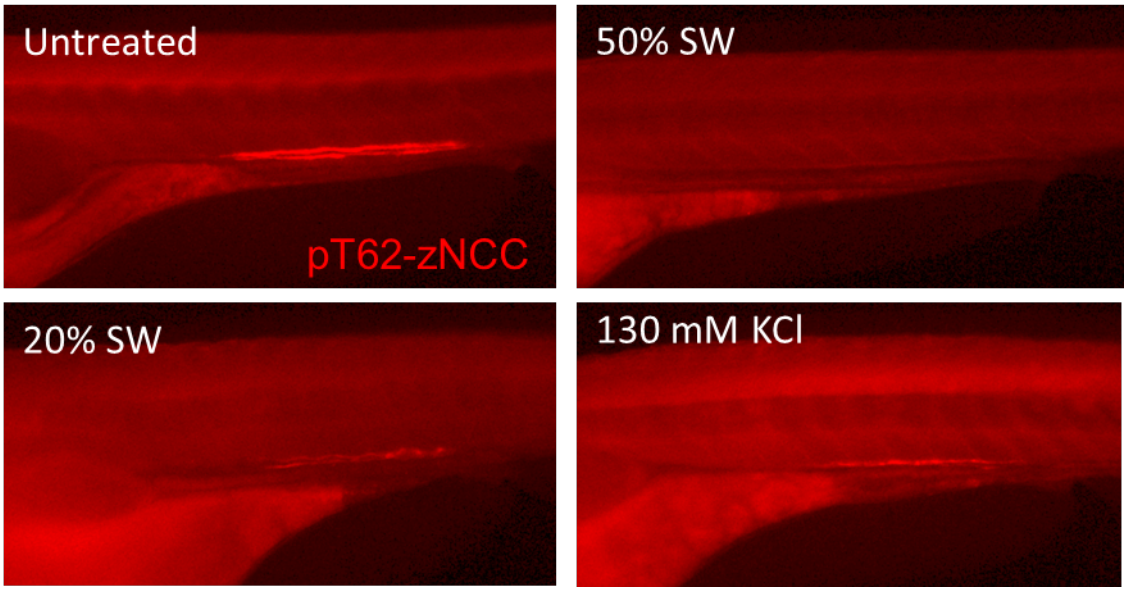


Fig. 2. Decrease in phosphorylation of zNCC in zebrafish larvae treated with increased salinity overnight. High concentration of KCl did not induce reduction in phospho-zNCC.

Modulation of phospho-zNCC by knockdowns of ppp1r1b

We recently identified protein phosphatase 1 inhibitor-1 (I1) as a novel regulator of NCC and DCT function in the mouse kidney [18]. The zebrafish *ppp1r1c* is the orthologue of the mouse I1, but *in situ* hybridization did not detect mRNA transcripts for *ppp1r1c* in the pronephros

(data not shown). Interestingly, however, a closely related homologue, *ppp1r1b*, was specifically expressed in the pronephric DL (Fig. 3A). Since ppp1r1b may have a similar function in the DL as does I1 in the mouse DCT, we performed morpholino knockdown experiments on *ppp1r1b*. Whole-mount immunostaining of phospho-zNCC in *ppp1r1b* morphants demonstrated reductions in the staining intensity compared to controls while there was no apparent change in mRNA abundance detected by *in situ* hybridization (Fig. 3B).

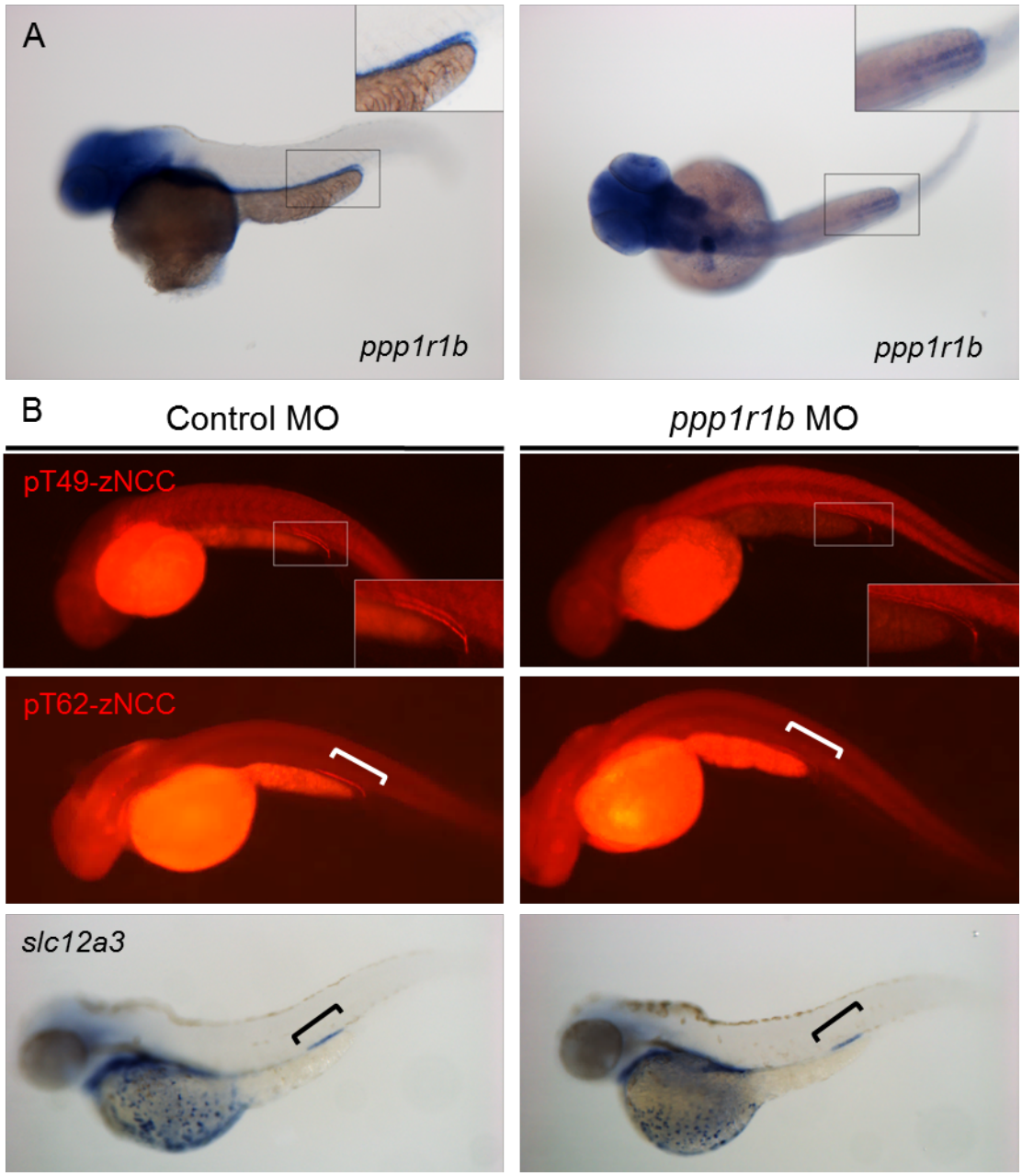


Fig. 3. (A) mRNA expression of the zebrafish *ppp1r1b* in the DL of the pronephros. Insets show a higher magnification of a region around the DL. (B) Decrease in phosphorylations of zNCC in *ppp1r1b* morphants. mRNA transcript abundance in *ppp1r1b* morphants is comparable to control.

4.5. Discussion

Our study presents a proof-of-concept for the use of the zebrafish as a new *in vivo* model system to study NCC phosphorylation and activity. The zebrafish orthologue of *SLC12A3* that encodes NCC is restricted to the DL both at the mRNA and protein levels. As in mammalian kidneys, the zebrafish NCC is also localized to the apical surface of the DL in the pronephros as well as in the mesonephros, suggesting that the function of NCC in NaCl reabsorption in the distal segment is conserved from zebrafish to humans. This is further supported by our result that high salinity treatments reduced the abundance of phospho-zNCC in the pronephros, analogous to what is seen with phospho-status of NCC in mice on high salt diets. Although involvement of NCC in NaCl regulation is likely conserved in the zebrafish, our observation that the excess K treatment did not induce a major change in phosphorylation of zNCC implicates that NCC regulation may differ in detail between the zebrafish and mammals.

In addition to environmental stimuli, we showed that zNCC activity can be genetically modulated in the zebrafish pronephros. Our previous work on the mouse DCT transcriptome identified I1 as a DCT-enriched gene that controls NCC activity, thereby contributing to the regulation of arterial blood pressure [18]. I1 is encoded by *Ppp1r1a* that comprises a family of genes that inhibit protein phosphatase 1 [19]. The zebrafish possess the orthologues of these inhibitors. Although the direct orthologue of I1 in the zebrafish is encoded by *ppp1r1c*, this gene does not exhibit apparent expression in the pronephros. Instead, another PP1 inhibitor, *ppp1r1b* is specifically expressed in the DL segment of the pronephros, of which functional knockdowns led to reduced phospho-zNCC. In mammalian kidneys, *Ppp1r1b,* encoding DARPP-32, is expressed in the TAL [20]. It remains to be determined whether DARPP-32 regulates NKCC2, a close homologue of NCC, in the TAL, in a similar fashon to the NCC regulation by I1 in the DCT, but our data present evidence that the regulatory mechanism of ion transporters by protein phosphatases may already exist in the zebrafish.

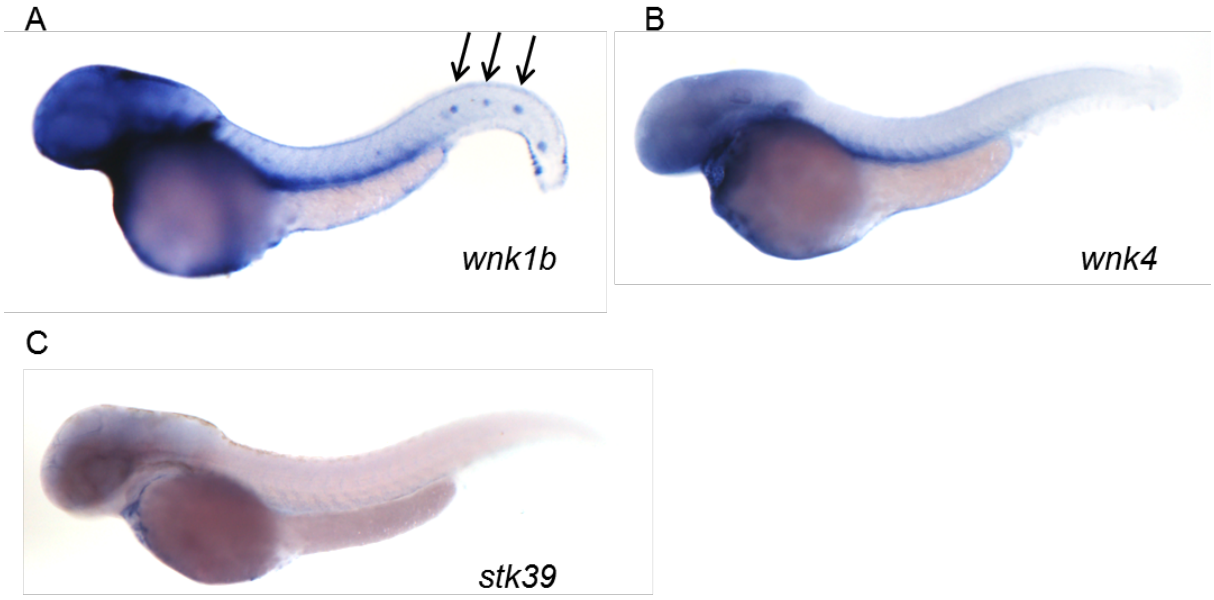
NCC activity and DCT function are regulated by several pathways involving kinases, such as SPAK and WNKs [21]. Notably, these major regulators of NCC are conserved in the zebrafish. Yet, to what extent regulatory mechanisms of NCC are conserved warrants further investigations. Of note, none of these orthologues exhibits evident expression in the zebrafish pronephros (Supplementary Fig. 1). This does not exclude that NCC is regulated through SPAK or WNK pathways in the pronephros as in the mammalian kidneys, but there appears to be variations in regulations of NCC between zebrafish and humans. Comparative analysis of DCT

transcriptomes among different species (e.g. zebrafish, mouse, human) would give us a clue on the degree of conservation, which should further prompt research into the complex network of NCC regulation using the zebrafish.

4.6. Outlook

One limitation of the antibodies we developed in this study is a poor outcome from Western blot analysis that should enable quantification of NCC phosphorylation. The results we have shown so far mainly based on immunofluorescence but the sensitivity of this technique is not appropriate for detection of slight alterations in protein abundance. Therefore, our observations that the several treatments did not cause changes in NCC activity in the zebrafish do not necessarily mean "no change". The future work should examine the phospho-status of zNCC with more quantitative ways, including Western blot analysis.

4.7. Supplementary figures



Supplementary Fig. 1. mRNA expression of the zebrafish orthologues of known regulators of NCC. (A) *wnk1b* shows a basal expression with some specific expression in neuromasts at 48hpf (arrows). (B) *wnk4* shows a basal expression around the whole embryo. (C) *stk39* encoding SPAK shows a weak basal expression at this stage of development.

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Chapter 5

Transgenic labeling of the late distal segments in the zebrafish kidney using the promoter from *slc12a3*

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Report on an ongoing research

Personal contribution:

Bioinformatics, construction of targeting vectors, microinjection, embryo screening, morpholino knockdowns, preparation of all figures and writing of the manuscript

5.1. Abstract

Transgenic approaches provide useful tools to dissect molecular mechanisms underlying organogenesis. Due to the optical clarity of embryos and the ease of transgenesis, the zebrafish is particularly suited to applications of transgenic technologies. *SLC12A3* encodes for thiazide-sensitive sodium chloride cotransporter (NCC) specifically expressed in the distal convoluted tubule (DCT) of the mammalian kidneys. The DCT plays a critical role in sodium reabsorption by the nephron and NCC is the major apical transport pathway of the DCT. One of the hindrances of NCC research is a lack of a specific promoter that drives expression only in the DCT in the kidney. Here, we show that a zebrafish 1 kb *slc12a3* promoter region is sufficient to drive restricted expression in the distal late segment of the zebrafish pronephros, the equivalent segment to the mammalian DCT. Furthermore, this transgene expression persists through to adulthood, allowing studies of development of the mesonephros from the pronephros. This transgenic zebrafish should facilitate detailed analyses of regulations of NCC and DCT function.

5.2. Introduction

The thiazide-sensitive sodium chloride cotransporter (NCC) is an electroneutral transporter localized to the apical membrane of epithelial cells lining the distal convoluted tubule (DCT) [1]. NCC is a major player in sodium reabsorption by the distal nephron. Two monogenic disorders, Gitelman syndrome (GS) and familial hyperkalemic hypertension (FHHt), caused by hypo- and hyper- activity of NCC respectively, exhibit syndromes with pronounced alterations in blood pressure and other ion balance, underlying the importance of this transporter in ion homeostasis [2]. Moreover, heterozygous carriers of *SLC12A3* that encodes NCC have a lower blood pressure and lower risk of hypertension than the rest of the population, suggesting that NCC may also contribute to more common forms of arterial hypertension [3].

In contrast to the pronephros in amniotes, the zebrafish pronephros is functional from an early stage of development [4]. The pronephric glomerulus filters the blood plasma and subsequently, the tubule modifies the filtrate to form urine. The zebrafish pronephric tubule is divided into different segments [5]. Except for the thin loop of Henle, the zebrafish pronephric tubule consists of the same functionally different segments as in the mammalian metanephric nephron [6]. For instance, the distal pronephros is composed of two segments, distal early (DE) and distal late (DL), which correspond to the mammalian thick ascending limb (TAL) and DCT, respectively. Significantly, the orthologous genes for the critical ion transporters, including NCC, in the TAL and DCT are also present in these equivalent segments in the zebrafish pronephros.

The ease of transgenesis in combination with the transparency of embryos and its external development, the zebrafish presents an ideal system for *in vivo* analyses of organogenesis and dissection of underlying molecular mechanisms [7]. Of particular importance, the zebrafish pronephros provides an easily accessible nephric tubule for direct functional investigations *in vivo*. In this study, we describe a generation of a transgenic zebrafish line with specific transgenic expression in the late distal segment using the promoter region of the zebrafish orthologue of *SLC12A3*. The established transgenic line stably expresses mCherry in the pronephric DL that persists through to adulthood. This transgenic zebrafish should facilitate studies of NCC regulation as well as DCT function and development.

5.3. Materials and methods

Bioinformatic analysis of the slc12a3 *promoter sequence*

The upstream region of *slc12a3* was aligned with the upstream region of *trpv4* to search for conserved non-coding sequences (CNS) by VISTA (http://lagan.stanford.edu/lagan_web/index.shtml) (Threshould; ≥70%, ≥100bp). The analysis included 6kb and 4kb upstream sequences of slc12a3 and trpv4, respectively, limited by the adjacent upstream genes.

Construction of the expression vector

The 1kb fragment of the promoter was amplified from WIK genomic DNA with Phusion High-fidelity DNA polymerase (Thermo Scientific). The primers contained attB4 and attB1 sequences at the 5`ends of the forward and reverse primers, respectively. The PCR product was first subcloned into the pDONR-P4P1R vector and then recombined into the pDestTol2CG2 vector with the mCherry middle element and the 3`-polyA element using the Gateway Vector Conversion kit (Invitrogen).

Transgenesis

Plasmid DNAs for microinjection were purified using miniprep (Macherey-Nagel, Oensingen, Switzerland). Tol2 transposase mRNA was *in vitro* transcribed using mMessage mMachine Sp6 kit (Life Technologies). The expression construct was co-injected into fertilized embryos at the one-cell stage with transposase mRNAs. The injected embryos were screened for *cmlc2*:eGFP marker and positive embryos were raised to adulthood. F0 founders were outcrossed to wild-type zebrafish and resulting F1 offsprings were then screened for mCherry expression in the DL. Positive F1 adults were subsequently outcrossed to wild-types to obtain the stable F2 generation.

Zebrafish husbandry

Zebrafish were maintained under a 14h/10h light/dark cycle. Fish were bred as previously described and the embryos were raised at 28 ^{0}C in E3 medium [8].

5.4. Results

Isolation of the zebrafish slc12a3 *promoter*

The zebrafish orthologue of *SLC12A3* shows a restricted expression in the DL segment of the pronephric tubule (Chapter 4). In order to determine whether the promoter region of the zebrafish *slc12a3* drives a specific transgene expression in the DL, we first compared the upstream sequence of *slc12a3* with that of *trpv4*. *trpv4* is also specifically expressed in the distal portion of the pronephros and therefore, common CNS, if any, in the regulatory regions of these two genes may harbor *cis*-regulatory elements that are essential for DL specific expression [9]. The VISTA search predicted two CNS within 4kb upstream region of *slc12a3* (Fig. 1A).

Approximately 1 kb upstream region, encompassing the most proximal CNS (CNS1), was assembled into the Tol2 reporter construct to assess whether this fragment drives mCherry reporter expression in the DL of the pronephric tubule (Fig. 1B). We could detect a mosaic expression of mCherry in the DL segment in the F0 embryos, suggesting that 1kb upstream region of *slc12a3* contains sufficient regulatory elements to drive the restricted expression of transgene in the DL of the zebrafish pronephric tubule (Fig. 1C).

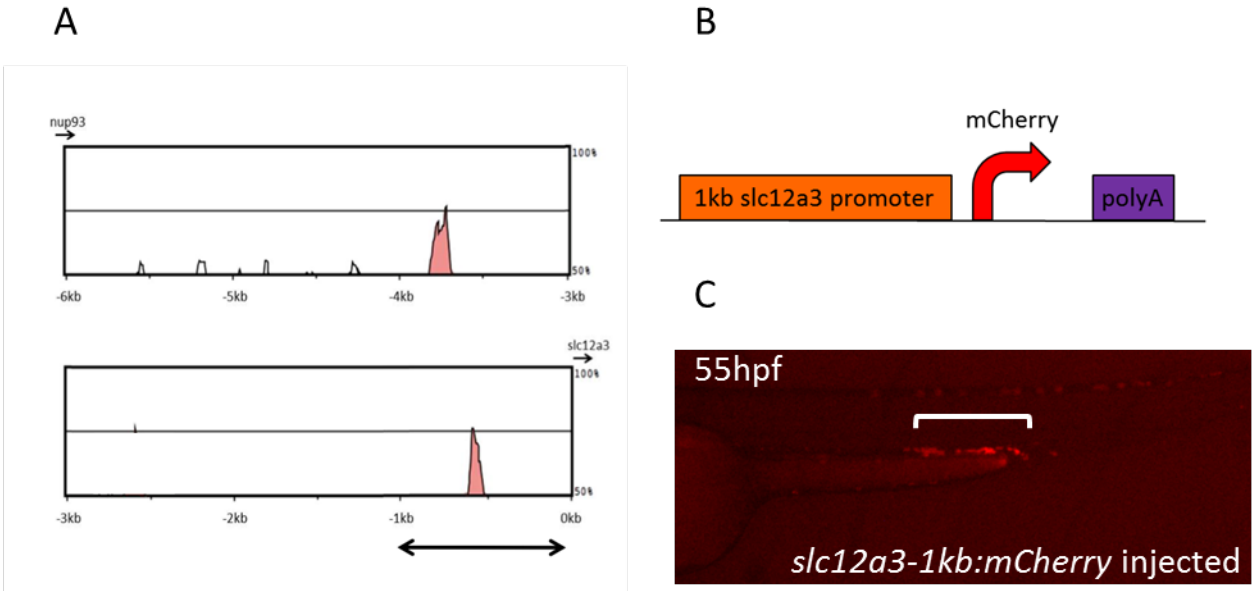


Fig. 1. (A) Conserved non-coding sequences (CNS) in the 6 kb upstream region of *slc12a3*. An approximately 1 kb upstream region (double arrow) containing one of the CNS was cloned into Tol2 constructs. (B) Targeting Tol2 vector with mCherry fused to a 1 kb fragment of slc12a3 putative promoter. (C) Transient expression of mCherry in the DL of the pronephros driven by the 1 kb promoter of *slc12a3.*

mCherry expression in Tg(slc12a3:mCherry)

Positive F1 individuals were crossed to wild-type zebrafish to establish stable Tg(*slc12a3*:mCherry). 50% of all the resulting F2 progenies were mCherry positive, indicating a single integration event of the *slc12a3:*mCherry transgene element into the genome. mCherry transgene expression was detected in the pronephric DL of the stable transgenic line, starting at around 36hpf and becoming uniform from 48hpf onward (Fig. 2). The mCherry expression in the DL extends toward the proximal tubule with development as the distal segment elongates [10]. We also monitored multiple generations of Tg(*slc12a3*:mCherry) (F4) and detected stable mCherry expression, suggesting that this promoter fragment is not particularly susceptible to transgene silencing.

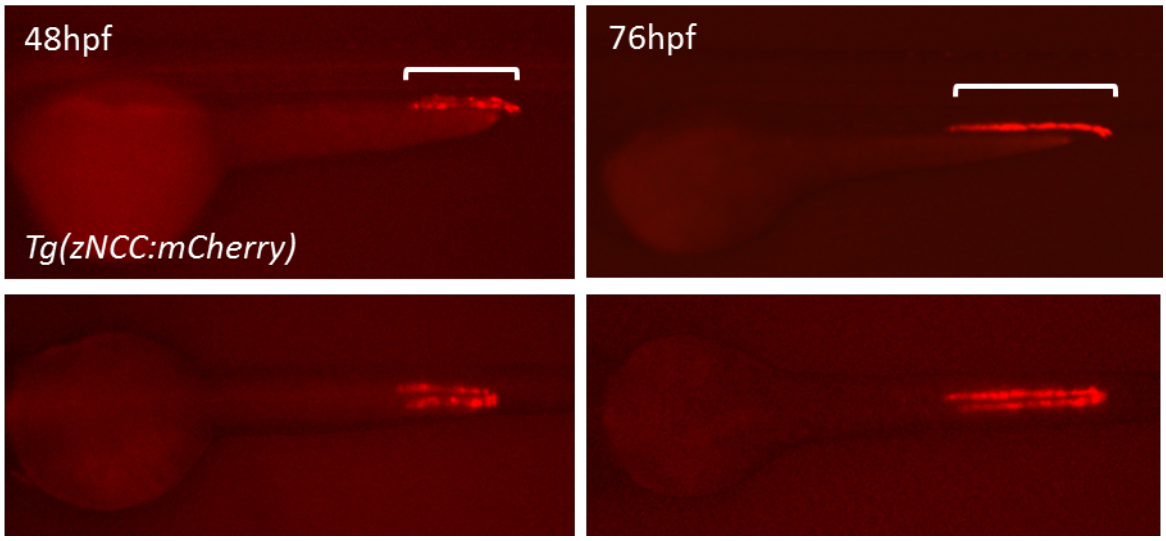


Fig. 2. *slc12a3*:mCherry expression in the DL (bracket) of the F1 stable transgenic fish. The mCherry labelled DL segments elongate with development.

*Development of the mesonephric nephron in Tg(*slc12a3*:mCherry)*

To determine whether the mCherry transgene is active also in the mesonephros, F2 adult zebrafish were dissected and the kidneys were examined by fluorescence microscopy. mCherry expression was visible in the distal portion of adult mesonephric nephrons (Fig. 3A). Additionally, mCherry transgene expression was also detected in the two major collecting ducts of the mesonephros (Fig. 3B). In order to define the development of the distal nephron in the mesonephros, we monitored the mCherry transgene expression from larval development through to a juvenile stage. The mCherry expression stays in the bilateral tubules until around

20 dpf, after which several clusters of mCherry positive cells appeared around the caudal region of the DE segment (Fig. 3C). At around 25 dpf, a mesonephric nephron started to grow on the rostral region of the pronephric DL (Fig. 3D). Several other nascent nephrons appeared along the DL toward 30 dpf.

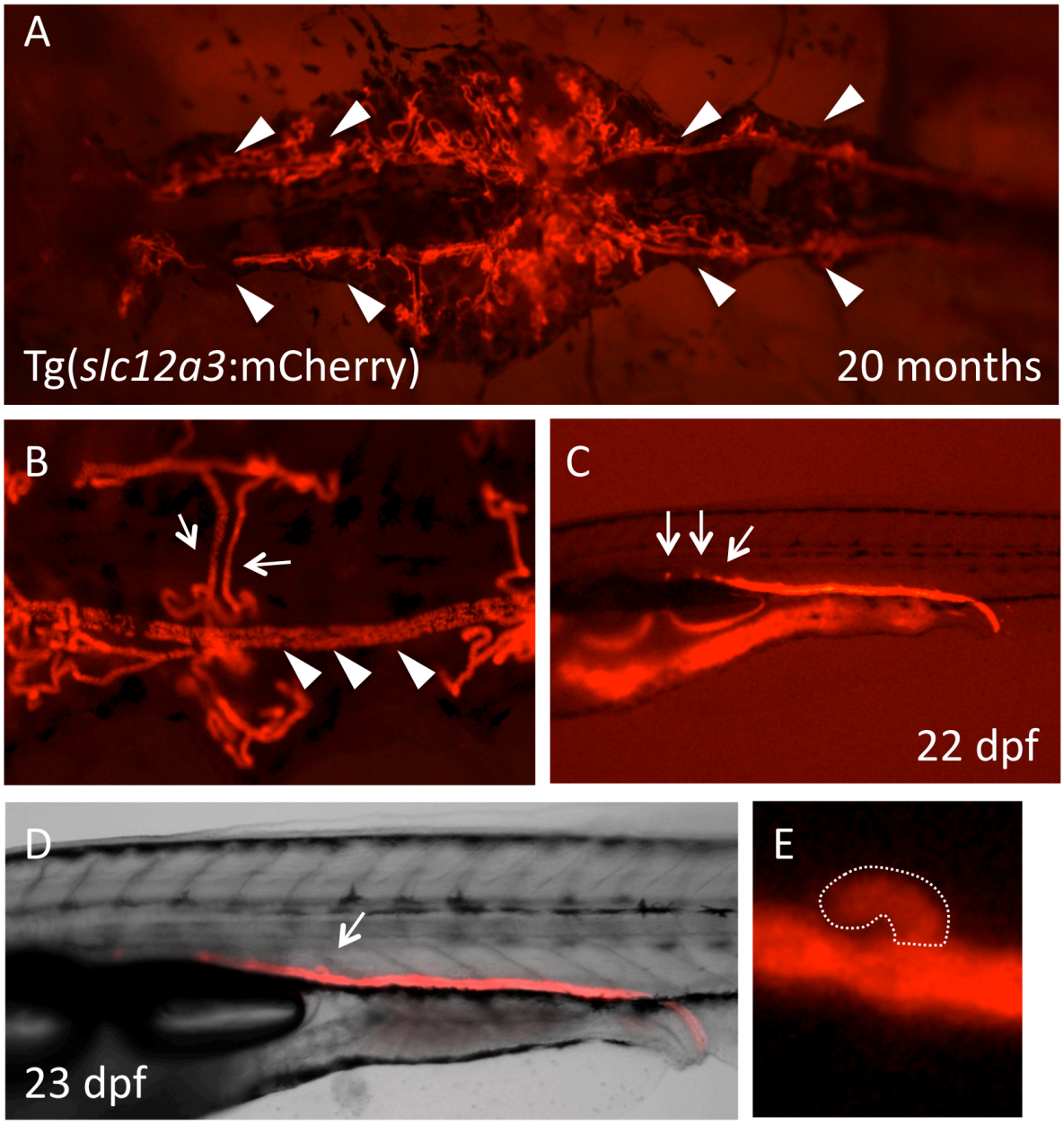


Fig. 3. Mesonephric kidney in Tg(slc12a3:mCherry). (A) Ventral view of the mesonephric kidney from a 20 months old Tg(slc12a3:mCherry). Nephrons are symmetrically organized around the two major collecting ducts (arrowheads). (B) A higher magnification of the mesonephric kidney. mCherry positive distal nephrons (arrows) are fused to the large collecting duct (arrowheads). (C) Lateral view of a 22 dpf juvenile. Clusters of mCherry positive cells start to appear around the caudal region of the DE segment (arrows). (D) Lateral view of a 23 dpf juvenile. A nascent nephron is observed on top of the rostral region of the pronephric DL (arrow). (E) A higher magnification of the nascent mesonephric nephron fused to the pronephric DL.

5.5. Discussion

Here, we demonstrate that 1kb upstream region of the zebrafish *slc12a3* drives a specific expression of mCherry in the DL segment that overlaps with the endogenous expression of *slc12a3*. Transgene expression in Tg(*slc12a3*:mCherry) starts to be visible at around 36 hpf while strong endogenous *slc12a3* expression is already found in the posterior portion of the developing pronephric tubules at 10-13 somite stages. This indicates that additional promoter elements that are not included in the 1kb upstream sequence likely participate in the early expression of *slc12a3* in the zebrafish pronephros. Although specific *cis*-elements responsible for the DL restricted expression remain to be identified, an *in silico* analysis (Supplementary methods) predicts several binding sites for known transcription factors in the CNS1 in the proximal promoter of the zebrafish *slc12a3*. These include canonical transcription factor binding sites, such as TATA box. The TATA box is frequently found in the promoters of tissue-specific genes while absent in the promoters of ubiquitously expressed genes [11]. Of particular interest here is the presence of the Evi1 binding site in the CNS1. Evi1, the ecotropic viral integration site-1, is a zinc finger transcription factor involved in myeloid leukemia [12]. A recent work demonstrated that the zebrafish *evi1* expression is restricted in the DL of the pronephros and more significantly, evi1 controls the segmentation of the distal pronephros during zebrafish development [13]. In consistent with this report, morpholino knockdowns of *evi1* led to a shortened transgene mCherry expression in Tg(*slc12a3*:mCherry), suggesting a possible involvement of this transcription factor in regulation of gene expression of *slc12a3* in the zebrafish pronephros (Supplementary Fig. 1).

We recently obtained a mouse DCT transcriptome from transgenic mice under parvalbumin (PV) promoter by using automated sorting of fluorescent tubules with COPAS (Complex Parametric Analyser and Sorter) [14]. Interestingly, this study shows that transcript abundance of *Evi1* transcription factor in the mouse DCT is 11 fold higher than the rest of the nephron, leading us to hypothesize that Evi1 may be an evolutionarily conserved transcription factor involved in regulations of DCT specific genes. Although LAGAN program did not detect any CNS in the proximal promoters of *Slc12a3* between zebrafish and mice, the *in silico* analysis did predict an evi1 transcription factor binding site at 166 bp upstream from the transcription start site of the mouse *Slc12a3*. Thus, we also tested a putative promoter activity of the proximal region of the mouse *Slc12a3* in zebrafish. The resulting F0 embryos injected with the Tol2 construct containing the 1 kb mouse *Slc12a3* promoter showed transgene expression in

the proximal tubule of the zebrafish pronephros (Supplementary Fig. 2). In addition, the transgene expression was observed also in the cardiac and skeletal muscles, and the blood progenitors. This result was somewhat surprising, but these organs are all derived from the mesoderm. Therefore, although this observation indicates that the mouse proximal promoter containing the putative Evi1 binding site may not be sufficient to confer a distal specificity of a transgene expression in the kidney, it implies that the proximal promoters of *Slc12a3* both in zebrafish and mice include conserved *cis*-regulatory elements that are necessary to drive gene expression in the mesodermal organs.

Non-coding sequences under high conservation pressure are hypothesized to contain regulatory elements that determine tissue specificity, timing and levels of expression of genes within the regulatory stretch [15]. In this respect, it is an interesting observation that LAGAN program did not retrieve any sequence conservations in the promoter regions of *Slc12a3* between the mouse and zebrafish, but yet, both regions directed transgene expression in the pronephros. This is anecdotal of a previous report that functional information is conserved in non-coding sequences below the detection level of *in silico* analyses [16]. Future work should specify *cis*-elements and transcription factor binding sites, and determine how they are arranged in the promoter regions of zebrafish and mouse *Slc12a3*. This would help us not only understand transcriptional networks that govern gene expression in the distal kidney but also how segment specificity of gene expression in the nephron is achieved and how it has evolved from lower vertebrates to mammals.

The *slc12a3* promoter isolated in this study is active throughout the zebrafish life cycle, which allowed us to monitor the transition of the distal nephron from the pronephros to the mesonephros. Recent two reports have presented evidence that the pronephric DE and DL serve as a "scaffold" on which new mesonephric nephrons grow and the pronephric distal segments later become the two major collecting ducts in the adult mesonephric kidney [17][18]. The *slc12a3*:mCherry transgene expression in juvenile zebrafish adds convincing evidence to this notion that the distal pronephric tubules are the origin of the mesonephric collecting ducts. It is of interest that the 1 kb *slc12a3* promoter is still active in the collecting ducts in addition to DL segments in the mesonephros while the endogenous slc12a3 is only expressed in the DL and absent in the collecting ducts of the adult kidney (data not shown). There are likely additional *cis*-elements outside the 1 kb region that restricts *slc12a3* gene expression only to the DL in the mesonephros. Knowledge on the mesonephros development

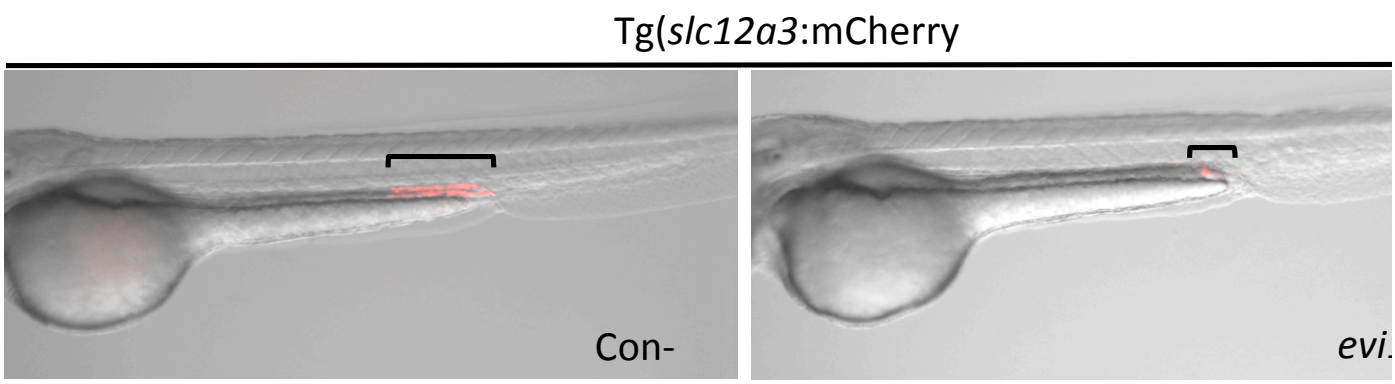
in the zebrafish is yet limited but the transgenic zebrafish we have established can also be exploited to analyze the morphogenesis of the zebrafish adult kidney.

In summary, we report the generation of Tg(*slc12a3*:mCherry) line with specific mCherry expression in the late distal segments both in the pronephros and mesinephros. The transgenic line can be used for a number of applications, including live imaging and isolation of DL specific cells. In addition, this transgenic zebrafish should be able to serve as a platform for pharmacological and genetic screens to identify chemicals and genes that modulate *slc12a3* expression, providing powerful tools for NCC and DCT research.

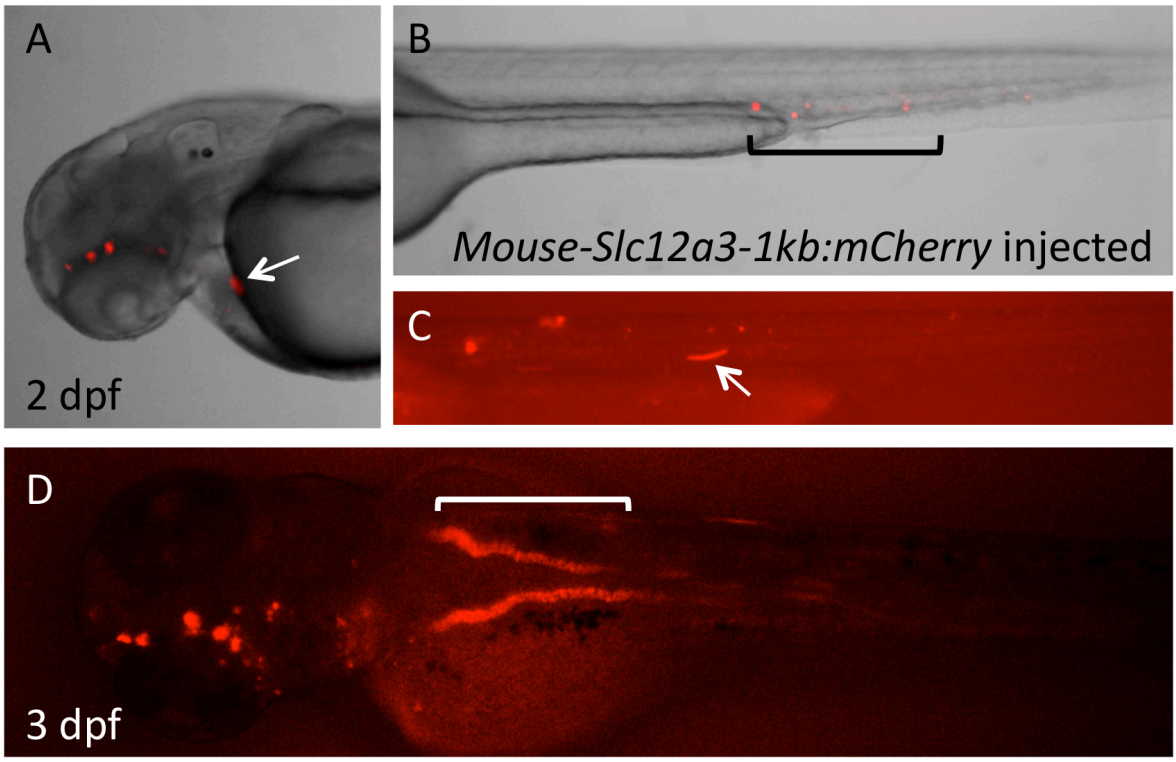
5.6. Outlook

Using mCherry transgene fluorescence as a marker, DL segments are currently microdissected from adult Tg(*slc12a3*:mCherry). RNAs are extracted from these isolated DL tubules for RNA-seq analysis to obtain a zebrafish DL transcriptome. Our recent mouse DCT transcriptomic analysis revealed that expressions of most of the regulators of NCC, such as SPAK and WNKs, are enriched in the DCT, raising a hypothesis that other DCT enriched genes may be potential new regulators of NCC. Indeed, this mouse DCT transcriptomic analysis led to an identification of protein phosphatase 1 inhibitor-1 (I-1) as a novel regulator of NCC and DCT function [14]. The critical transporters in the distal nephron are conserved during evolution. Therefore, it is reasonable to speculate that important NCC regulators are already present in the zebrafish DL, which might be identified by comparing the mouse DCT and the zebrafish DL transcriptomes. The human DCT is also available and this will also be used to identify evolutionarily conserved regulators of NCC [19]. Once candidate genes for such regulators are isolated, functional analysis will be performed based on morpholinos and/or CRISPR/Cas9 system and NCC activity will be assessed using the antibodies described in chapter 4. Causative genes for some human patients with hereditary forms of GS and FHHt are yet unknown. Our evolutionary approach may help us identify novel genes, of which disruption causes these tubulopathies.

5.7. Supplementary figures



Supplementary Fig. 1. Morpholino knockdowns of evi1 reduced the length of mCherry expressing DL segment at 2 dpf.



Supplementary Fig. 2. Promoter activity of the 1 kb upstream region of the mouse *Slc12a3*. Transient expression of mCherry transgene is observed in the heart (A; arrow), blood progenitors (B; bracket) and skeletal muscles (C; arrow) at 2 dpf. (D) mCherry transgene appears in the proximal pronephros at 3 dpf.

5.8. Supplementary methods

In silico *analysis of putative transcription factor binding sites*

Putative transcription factor binding sites were searched using TFSEARCH program (http://diyhpl.us/~bryan/irc/protocol-online/protocol-cache/TFSEARCH.html).

Morpholino knockdowns

Two published morpholinos targeted to the splice donor site of exon 3 (5`-CTGAGTGACTTACATATGAAGGGCT-3`) and the splice acceptor site of exon 4 (5`-TTGTGGCAGACCTCACGACGGTGTT-3`) of *evi1* were co-injected as previously described into Tg(*slc12a3*:mCherry) at 1-4 cell stages [13].

5.9. References

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Chapter 6

General discussion

In the studies presented here, we took advantage of the zebrafish model in order to elucidate mammalian renal function. The studies consist mainly of two distinct parts; the glomerulus (chapter 3) and the distal tubule (chapter 4 & 5). In the glomerulus part, we exploited the well-conserved pronephric glomerulus to identify novel genes involved in the filtration function of the glomerulus, which are potentially relevant to pathogenesis of human nephrotic syndrome (NS). In the distal tubule part, we explored the use of the zebrafish to analyze molecular mechanisms underlying NaCl homeostasis through NaCl cotransporter (NCC) in the distal kidney. In the following section, we will summarize and discuss obtained results and lay out future perspectives of renal research using the zebrafish.

6.1. The glomerulus

6.1.1. IQGAP2: A new player in podocyte biology

In the first part of the thesis, we used datasets from transcriptional analyses of human kidney biopsies and subsequently analyzed function of candidate genes in the zebrafish. Comprehensive transcriptional profiles are a useful and very informative resource to investigate organs, regions and cell populations of interest. However, comprehensive approaches typically yield a large amount of dataset, which renders isolation of target genes laborious and time-consuming in the rodent models. Moreover, *in vitro* system using cultured human podocytes can achieve rapid assessments of gene function with a cost effective manner, but however, it is often difficult to recapitulate the native state of this highly specialized epithelial cell in cultured system. In addition, the three components of the glomerular filtration barrier (GFB) are interdependent, and thus *in vitro* systems are not appropriate in modeling the podocyte in the proper physiological context. The zebrafish circumvents these limitations by providing an accessible *in vivo* model system. Our approach allowed us to identify a Rho-GTPase activating protein, IQGAP2, as a gene essential for maintenance of the podocyte integrity. IQGAP2 is enriched in the glomerulus of the human kidney, downregulated in human NS and its functional knockdown in zebrafish led to the phenotypes that are similar to the characteristic features of

NS. This study suggests that downregulation of IQGAP2 may be relevant to pathogenesis of NS in humans.

6.1.2. IQGAPs in regulation of the podocyte actin cytoskeleton

IQGAPs are evolutionarily conserved proteins in eukaryotes. IQGAPs are scaffolding proteins that mediate formation of protein complexes, involved in numerous cellular processes [1]. A homologous protein is already present in the yeast *Saccharomyces cerevisiae* (Iqg1p/Cyk1p) [2]. Roles of IQGAPs in cellular processes associated with the actin cytoskeleton, such as cytokinesis, are highly conserved from fungi to mammals. For example, IQGAPs play an essential role in the assembly of a contractile ring that generates a mechanical force to separate parent and daughter cells during cytokinesis and loss-of-functions of fungal IQGAPs result in the formation of multinucleated cells [3]. In mouse oocytes and embryos, IQGAP1 is found at the contractile ring during cytokinesis [4]. IQGAPs have been well characterized at the cellular level, but however, their physiological relevance at the organismal level has only begun to be revealed [5].

We presented the first evidence that IQGAP2 plays an essential role in maintenance of the podocyte integrity. Most vertebrates possess three isoforms of this protein, IQGAP1, IQGAP2 and IQGAP3 [6]. They are similar in structure, but these isoforms exhibit different tissue distributions. In mammals, while IQGAP1 is ubiquitously expressed, IQGAP2 is predominantly expressed in the liver and IQGAP3 is expressed in mainly in the brain [7][8]. Significantly, the functional domains involved in regulations of the actin cytoskeleton exhibit a high degree of conservation. Calponin homology domain (CHD) and binds to the F-actin and GAP-related domain (GRD) interacts with small GTPases that are known to regulate the actin cytoskeleton [9]. These two domains are especially well conserved among the isoforms as well as among different vertebrate species. Since the elaborate structure of podocytes is maintained by the dynamic architecture of the actin cytoskeleton, it is very likely that IQGAP2 contributes to the barrier function of podocytes through modulation of the actin cytoskeleton [10].

It is of particular note that the closely related protein, IQGAP1, has been demonstrated to interact with Nephrin and other slit diaphragm (SD) components linked to the actin cytoskeleton [11][12]. Although function of IQGAP1 in podocytes *in vivo* is yet to be revealed, direct associations of IQGAP1 with SD component suggest that IQGAP1 may regulate the podocyte

integrity through modulation of cytoskeletal dynamics [13]. We need more work to determine whether IQGAP1 and IQGAP2 possess redundant function in regulating the podocyte cytoskeleton or each of them plays a unique role in maintaining the actin cytoskeleton. Of interest, IQGAP1 has been demonstrated to form homo-oligomers to activate the small GTPase, Cdc42 [14]. Although formation of hetero-oligomers of IQGAP1 and IQGAP2 have not been reported, considering their sequence similarity, it would be an interesting speculation that IQGAP2 may form hetero-oligomers with IQGAP1 to modulate the regulation of the actin cytoskeleton, which then maintains the podocyte barrier function. Identification of interacting partners of IQGAP2 in podocytes would help us clarify detailed mechanisms by which IQGAP2 maintains the podocyte integrity.

6.1.3. Ca^{2+} signaling in podocytes and acquired NS

Ca^{2+} signaling controls various cellular functions, including remodeling of the actin cytoskeleton through modulation of Rho-GTPases [15]. In podocytes, TRPC5 and TRPC6, members of the transient receptor potential superfamily, are the two players so far identified to regulate intracellular Ca concentration [16][17]. TRPC6 associates with the actin cytoskeleton and is known to be connected to the SD through binding to Nephrin and Podocin, modulating Ca entry locally at the SD. The importance of these Ca^{2+} channels and Ca^{2+} signaling in podocytes is evidenced by familial cases of focal segmental glomerulosclerosis resulting from genetic alterations in TRPC6 activity [18]. Changes in Ca^{2+} concentration are sensed by calmodulin and signals are transmitted via calmodulin binding proteins [19]. In this respect, the function of IQGAPs as a mediator of Ca^{2+}/Calmodulin signaling in cellular processes is worth a comment [20]. In fact, IQ motifs of IQGAPs interact with calmodulin and it has been reported that Ca^{2+} increases the affinity of calmodulin to IQGAP1 [21]. Moreover, a recent report has demonstrated that IQGAP1 expression is upregulated by a vasoactive hormone, angiotensin II, in rat glomeruli *in vivo* [22]. Angiotensin II modulates podocyte morphology by influencing intracellular Ca^{2+} concentration [23]. Therefore, it is tempting to speculate that IQGAP1 and IQGAP2 may be mediators of Ca signaling in podocytes, detecting changes in Ca^{2+} concentration and transmit the signals to downstream targets, Rho-GTPases, to modulate the cytoskeletal network.

A previous study showed an association of increased expression levels of TRPC6 and the acquired NS [24]. Furthermore, patients with hereditary NS carrying mutations in TRPC6 develop disease symptoms at a relatively late stage compare to the other forms of hereditary NS [17]. This permits an assumption that, unlike mutations in Nephrin or Podocin that result in severe forms of congenital NS, TRPC6 dysfunction may cause only slight alterations in podocyte function, which require time and other cues to lead to manifestation as NS. Since little is known about pathogenesis of acquired forms of NS, further studies on Ca signaling underlying the podocyte morphology and function with a possible involvement of IQGAP2 should be of significance. For such studies, transgenic zebrafish with genetically coded Ca^{2+} indicator provides a useful system to investigate Ca^{2+} signaling by visualizing intracellular Ca. This would contribute to a deeper understanding of how podocytes are structurally and functionally maintained, which may also give us insights into yet elusive mechanisms of pathogenesis of acquired NS.

6.2. The distal nephron

6.2.1. Conservation of NCC function in zebrafish

Research into body fluid regulation has attracted the intense attention. This is not only due to its clinical significance but also to the general physiological importance since osmoregulation is crucial in surviving in all habitats that experience perpetual disturbances, which challenge the physiology of any animals living therein [25][26]. Vertebrates live in a diverse range of habitats from extremely hypo-osmotic environment (fresh water) to arid deserts [27]. Accordingly, vertebrates are equipped with regulatory mechanisms to keep the physiology of the internal environment constant. It is striking that, although vertebrates occupy such a broad range of habitats with different physiological demands, they maintain ionic and osmotic parameters of the blood plasma very similar [28]. In fact, regardless of habitats, the osmolarity of the blood plasma and extracellular fluid is kept at approximately one-third of seawater [29].

In terrestrial life, the biggest physiological challenge is to keep water and ions, particularly, Na and Cl. Several transporters expressed in the distal segments of the kidneys play a major role in retention of NaCl [30]. In the second part of the thesis, we focused on NCC in the distal convoluted tubule (DCT) of mammalian kidneys and explored the use of the zebrafish pronephric

tubule to analyze the function of this transporter. The general role of the distal nephron in NaCl recovery is fundamentally conserved among vertebrates as exemplified by the case of some marine fishes that have lost the distal nephron during evolution likely due to lack of necessity to reabsorb NaCl in hyperosmotic environments [31]. Our observation in the high salinity treated zebrafish is consistent with this notion and presents evidence that the function of the distal nephron is, at least in part, supported by the activity of NCC.

6.2.2. Hormonal control of NaCl homeostasis in teleosts

Not all but many acute changes in ion transporter activities are initiated by neuroendocrine and endocrine cues [32]. A neuroendocrine hormone, arginine vasopressin (AVP), and aldosterone produced in the adrenal cortex are major hormones that function to keep water and Na in mammals. AVP stimulates Na reabsorption through NCC in the DCT [33]. In teleosts, AVP has been reported to stimulate vasoconstriction, thereby increase blood pressure [34]. The hypothalamus in the zebrafish brain expresses the orthologue of mammalian AVP [35]. We performed knockdown experiments on the zebrafish AVP and subsequently, analyzed NCC activity. However, functional knockdowns of endogenous AVP did not lead to apparent changes in NCC phosphorylation in the zebrafish pronephros (data not shown). Furthermore, we also treated zebrafish larvae with excess AVP but this did not induce changes in NCC activity as well. Although detailed experiments have to be followed to definitively conclude, it is reasonable to speculate that since fresh water fishes do not need to actively retain water, there may be little selective pressure on development of such function for AVP. It is generally accepted that extant vertebrates have evolved from an ancient fresh water fish [36]. Therefore, the original function of AVP may have simply been that as vasoconstrictor and its water and Na preserving function via NCC in the kidney may have added later during evolution.

The renin-angiotensin-aldosterone system (RAAS) constitutes one of the most important regulatory systems of body fluid in mammals [37]. Aldosterone, acts through mineralocorticoid receptor (MR) on the late DCT to stimulate NaCl reabsorption [38]. Aldosterone is present in all tetrapods, but is absent in teleosts [39]. In fact, there is yet no clear distinction between glucocorticoid and mineralocorticoid in teleost species and cortisol functions as ligand both for glucocorticoid receptor (GR) and MR [40]. Teleosts possess the orthologous genes for mammalian renin and angiotensin II that are involved in volume regulation [41]. For instance,

marine fishes cope with the challenge of constant dehydration by drinking surrounding water and subsequent secretion of excess ions, but this drinking behavior is initiated by angiotensin II [42]. Since the lungfish and amphibian synthesize aldosterone, the RAAS likely evolved when vertebrates started living on land [43]. Deciphering hormonal networks that control Na and water regulations in zebrafish would further facilitate the use of this animal for studies of activity of NCC and DCT function. This should not only further establishment of this animal model to study NCC function but also provide insights into the evolution of the intricate networks of regulatory mechanisms of body fluid homeostasis in humans, which cannot be revealed by studying only a few mammalian species.

6.3. The potential of the zebrafish model

6.3.1. Drug screening platform

Drug discovery is one of the most important challenges in medical research. *In vitro* systems achieve efficient screening of candidate compounds, but *in vitro* assays do not reliably prove therapeutic benefits *in vivo* [44]. Consequently, these screens are primarily designed for assays of compounds with slight modifications on similar therapeutic targets, bringing little promise to discover new compounds that exert beneficial effects in diseases without predefined molecular targets. Ideally, candidate molecules are best screened in the whole animal, but its application to the mouse model is labor-intensive and time-consuming. High fecundity and the external development make the zebrafish ideal for chemical screening. A large number of chemicals can be tested on zebrafish embryos in multiwell plates [45]. Recently, a small molecule was discovered through such screens to amplify hematopoietic stem cells in zebrafish [46]. This chemical, prostaglandin E2, has been successfully demonstrated to increase blood stem cells in mice and now it is in phase II clinical trial to treat human leukemia patients. Another example is the histone deacetylase inhibitor that was identified through small molecule screening [47]. Administration of this compound attenuated the pathological phenotype of zebrafish and mouse model of acute kidney injury, which would be expected to exert a similar therapeutic effect to treat human patients. As represented by these examples, the zebrafish presents a promising platform for discovery of new drugs that overcomes limitations of conventional approaches.

6.3.2. The use of the adult zebrafish

In contrast to the larval zebrafish, the adult zebrafish has attracted a relatively little attention as a model system. This is mainly due to the lack of tools that allow for investigations of gene function through to adulthood. For example, knockdown effects of morpholinos are limited within 5 dpf, after which they are too diffuse to exert their effects. The embryonic and larval zebrafish are a useful system but because of the very simplicity that makes it useful poses a challenge in modeling more complex human diseases. In the case of the pronephros, as it consists of only two nephrons, renal damage usually causes lethality of the zebrafish. Furthermore, the more complex, final form of the kidney, mesonephros, starts to develop approximately after 12 dpf. The technological advancement of targeted mutagenesis approaches, however, achieves analyses of gene function later stages of development through to adulthood. With constitutive knockout lines, it is possible to analyze gene function at the most appropriate stages when genes play the most critical role in a life cycle. This should expand the potential of the zebrafish model for human disease since many diseases are adult onset and age-dependent. Implementation of Cre/loxP system for inducible gene knockout approaches would add a further strength to the utilization of the adult zebrafish. Furthermore, techniques for physiological measurements used in mice should, in principle, be applicable to the adult zebrafish. Such approaches would lead to a more detailed characterization of genetics and physiology of the adult zebrafish, which then would pave a way for even more versatile applications of this animal model for research into human disease.

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IV CURRICULUM VITAE

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PUBLICATIONS

Sugano Y, Neuhauss SCF: Reverse genetics tools in zebrafish: a forward dive into endocrinology. *Gen. Comp. Endocrinol.* 188: 303–8, 2013

Sugano Y, Lardelli M: Identification and expression analysis of the zebrafish orthologue of Klotho. *Dev. Genes Evol.* 221(3): 179–86, 2011

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GRANTS

Comparative analysis of gene expression in zebrafish and mouse renal distal tubules to identify evolutionarily conserved regulators of thiazide-sensitive NaCl cotransporter [Forschungskredit, Faculty of Medicine, University of Zurich (55,000 CHF, 11 months, 2014)]

Establishing the zebrafish pronephros as an easy-to-use *in vivo* model to study the regulation of the renal thiazide-sensitive NaCl cotransporter [Hartmann Müller-Foundation (12,000 CHF, 1 year, 2012)]

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V ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my two main supervisors, Stephan Neuhauss and Jan Loffing. Without their valuable supports and fruitful discussions with them, I would not have made it to the degree. From these two excellent mentors, I have learned not only science but also how to be a scientist.

I would also like to thank Clemens Cohen for his useful advises on the course of development of the PhD projects. Maja Lindenmeyer in Cohen group for her help and support with the glomerulus project. Without their supports, together with initial work by Ines Auberger, I would not have made the project into publication.

Urs Ziegler has to be thanked as well. I am indebted to him and the people at the center of microscopy and image analysis for many of the imaging work. In particular, I would like to thank Txema Mateos, Therese Bruggmann, Bruno Guhl and Gery Barmettler for their help with electron microscopy. People in Adrew Hall group are also thanked for our occasional kidney imaging meetings.

I would like to thank the external committee of my PhD thesis, Iain Drummond, for his inspiring communications and all his work that has guided me through the research of the zebrafish kidney.

Special thanks go to the current and former members of Neuhauss group; especially, Kara for her great fish care and technical supports; Matthias for giving me practical advises on several techniques; Chiara and Tanja for being my friends of the zebrafish kidney group; Irene Hofmann, Marion, Edda, Lucia, Jingjng, Ruxandra, Thomas, Kaspar, Sabina, Fränzi, Steffi, Andre, Alex, Peter, Stella, Irene from Madrid, Röbi, Jenny, Andi, Valentine, Manu, David, Pascal, Bianca, Kulsum, Martin, Anja.

More thanks have to go to the members of Loffing group in the present and past; in particular, Dominique for her help with the mouse care and technical supports; Abhi for his help with mouse experiments; Nicole, Monique, Mads, Solveig, Claudia, Jan Czogalla, David, Aga, Katja, Marian, Marianna, Michelle, Twinkle; Also Nourdine for being a lively part of our group.

I would also like to thank my former mentor, Michael Lardelli in Adelaide, South Australia. His encouraging words made me decide to follow the path to step up onto the PhD study here in Europe. Without him, I am not sure if I had ever been here for this wonderful opportunity to develop as a scientist.

Two supervisors of my undergraduate study when I was still in Japan; Shugo Watabe and Shigeharu Kinoshita. Without their encouragement and help, I might not even have left the country to follow my passion to study abroad.

Lastly, I would like to thank my family in Japan and friends here in Switzerland as well as friends all around the world. I would not have made it here without them.